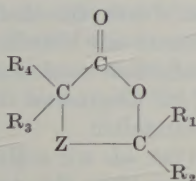


Beiträge zur Chemie α -substituierter Sulfide. V*

Über in 3-Stellung substituierte 4-Thiaisochroman-1-one

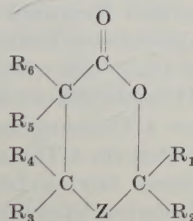
Von ALEXANDER SENNING und SVEN-OLOF LAWESSON

γ - und δ -Laktone, die im Ring ein Stickstoff-, Sauerstoff- oder Schwefelatome enthalten, sind nach einer Reihe verschiedener Methoden dargestellt worden und wurden auch für praktische Verwendungszwecke (Pflanzenschutzmittel, hydraulische Flüssigkeiten) vorgeschlagen (Völtzkow und Liebermann [1, 2] Bistrzycki und Brenken [3], Holmberg [4, 5], Fredga [6], du Pont (Patent) [7]).



I

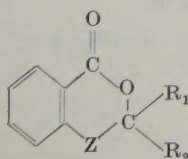
(Z = N, O, S)



II

(Z = N, O, S)

Mit einem Benzolring kondensierte Sechsringe vom Typ (III) sind, insbesondere



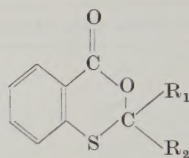
III

(Z = N, O, S)

in neueren Arbeiten, mehrfach im Zusammenhang mit Untersuchungen auf dem Gebiet der Pflanzenschutzmittel bzw. Pharmazeutika synthetisiert worden (Wallach [8], Borsche und Berckhout [9], Böseken [10], Calvet und Carnero [11], Renson und Schoofs [12], Kaufmann und Rossbach [13], Böhme und Schmidt [14], Mowry, Yanko und Ringwald [15, 16], Monsanto (Patent) [17, 18, 19], Senning und Lawesson [20, 21, 22]).

Zur Darstellung der erstmals von Mowry [17] als Insektizide vorgeschlagenen in 3-Stellung substituierten 4-Thiaisochroman-1-one (IV), denen unsere Untersuchungen

* IV. Mitteilung siehe Literaturzitat [22].



IV

3- R_1 -3- R_2 -4-Thiaisochroman-1-on
(2- R_1 -2- R_2 -1,3-Benzthioxan-4-on)

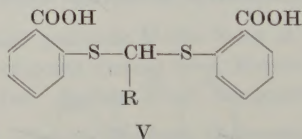
in der vorliegenden und mehreren früheren Mitteilungen galten [20, 21, 22], sind eine Reihe verschiedener Methoden angewandt worden.

Kaufmann und Rossbach [13] erhielten aus Thiosalizylsäure und symmetrischem Phthalylchlorid, das hier vermutlich in der unsymmetrischen Form reagiert, ein Spiroderivat des 4-Thiaisochroman-1-ons. Andere reaktive gem-Dihalogenide sind unseres Wissens noch nicht mit Thiosalizylsäure umgesetzt worden.

Böhme und Schmidt [14], die aus Thiosalizylsäureamid mit Aldehyden bzw. Ketonen Dihydro-1,3-benzthiazinderivate gewannen, erhielten im Falle des Chlorals unerwarteterweise das entsprechende 4-Thiaisochroman-1-on-derivat, das sie dann auch direkt aus Thiosalizylsäure und Chloral darstellten.

Mowry, Yanko und Ringwald [15] erhielten in der durch Quecksilberazetat und Schwefelsäure katalysierten Reaktion von Thiosalizylsäure mit Vinylazetat 3-Methyl-4-thiaisochroman-1-on. Aus den Diazetaten aromatischer und α,β -ungesättigter Aldehyde und Thiosalizylsäure konnte Mowry [16] in Gegenwart von Essigsäure und Schwefelsäure 4-Thiaisochroman-1-on-derivate darstellen.

Wir selbst erhielten ein 4-Thiaisochroman-1-on-derivat, als wir *o*-Methylmerkapto-benzoesäure in einem inerten Lösungsmittel chlorierten [20]. Bei der durch Chlorwasserstoff katalysierten Reaktion von Aldehyden mit Thiosalizylsäure in siedendem Eisessig konnten wir neben Alkyliden-bis-(*o*-karboxyphenylsulfiden) (V) ebenfalls 4-Thiaisochroman-1-on-derivate isolieren [21]. Bessere Ausbeuten an 4-Thiaisochroman-1-on-derivat wurden in Abwesenheit von Chlorwasserstoff erzielt [22].



V

Im weiteren Verlauf unserer Untersuchungen entwickelten wir eine Methode, die mit einer grossen Zahl von Aldehyden in guter Ausbeute 4-Thiaisochroman-1-on-derivate liefert: Aldehyd und Thiosalizylsäure werden zusammen mit einer katalytischen Menge *p*-Toluolsulfonsäure in siedendem Benzol umgesetzt, wobei das gebildete Wasser azeotrop abdestilliert wird [22]. Bei basischen Reaktanten empfiehlt es sich, etwas mehr als die äquivalente Menge *p*-Toluolsulfonsäure zu verwenden. Unsere Methode versagte in den bisher von uns geprüften Fällen nur beim Paraformaldehyd und beim Paraldehyd. Man erhält jedoch in beiden Fällen das 4-Thiaisochroman-1-on-derivat, wenn man die Reaktion in siedendem Eisessig in Abwesenheit von Chlorwasserstoff durchführt. Während wir in unserer letzten Mitteilung [22] über Umsetzungen mit aliphatischen Aldehyden berichteten, konnten wir in der vorliegenden Arbeit zeigen, dass unsere Methode sowohl mit neutralen als auch basisch oder

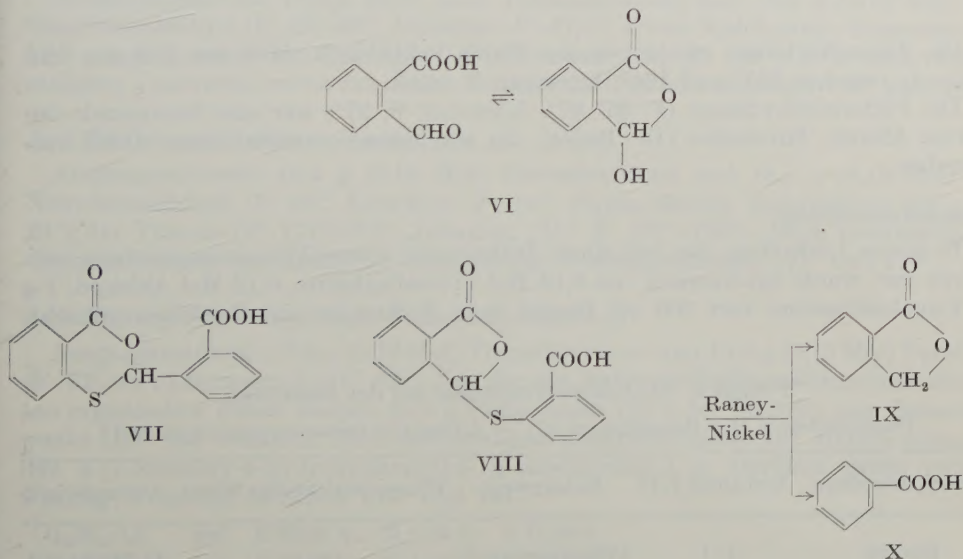
sauer substituierten aromatischen Aldehyden, mit Dialdehyden als auch mit empfindlichen heterozyklischen Aldehyden 4-Thiaisochroman-1-on-derivate liefert.

Die in einem amerikanischen Patent [7] beschriebene Methode, aliphatische Merkaptoensäuren unter der Einwirkung von wasserfreiem Natriumazetat in Ätherlösung bei Zimmertemperatur mit Perhalogenketonen zu kondensieren, gelingt mit Thiosalizylsäure und Aldehyden nicht.

Von den bisher weniger untersuchten verallgemeinerungsfähigen Darstellungsmethoden für 4-Thiaisochroman-1-on-derivate gedenken wir die Umsetzung von Thiosalizylsäure mit reaktiven Ketonen sowie mit gem-Dihalogeniden und die Chlorierung geeignet substituierter *o*-Alkylmerkaptobenzoesäuren in Kürze näher zu studieren.

Alle von uns dargestellten Verbindungen werden zur Zeit auf ihre biologische Aktivität geprüft. Über die Ergebnisse dieser Prüfung wird an anderem Ort berichtet werden.

Bei der Umsetzung von Thiosalizylsäure mit der in zwei tautomeren Formen existierenden Phthalaldehydsäure (VI), deren azyklische Form zum 4-Thiaisochroman-1-on-system (VII) und deren zyklische Form zum Phthalidderivat (VIII) führen sollte, konnten wir feststellen, dass die Reaktion ausschliesslich zum Phthalid-system führt. Die Struktur des Reaktionsproduktes ergab sich einerseits aus dem Infrarotspektrum, das mit dem von Wheeler, Young und Erley [23] bei ähnlich substituierten Phthalidderivaten beobachteten gut übereinstimmte (während die für δ -Laktone charakteristische Carbonylbande bei $5,80 \mu$ fehlte) und andererseits aus dem Abbau mit Raney-Nickel, der Phthalid (IX) und Benzoesäure (X) lieferte.



Beschreibung der Versuche

Alle Schmelzpunkte sind unkorrigiert. Die Infrarotspektrogramme wurden mit einem Spektrographen Perkin-Elmer Modell 137 aufgenommen. Die Analysen wurden im Institut für Analytische Chemie der Universität Uppsala ausgeführt.

Tabelle 1. Darstellungsmethoden für 4-Thiaisochroman-1-on-derivate.

Komponente I	Komponente II	Autoren	Bemerkungen
Thiosalizylsäure	Alkenylazetat	Mowry, Yanko und Ringwald [15]	Nur beschrieben für Vinylazetat, Katalysator: Quecksilberazetat, Schwefelsäure
Thiosalizylsäure	Aldehyddiazetat	Mowry [16]	Gelingt nur mit den Diazetaten aromatischer und α,β -ungesättigter Aldehyde, Katalysator: Essigsäure, Schwefelsäure
Thiosalizylsäure Thiosalizylsäureamid	Chloral	Böhme und Schmidt [14]	Spezialfall, unkatalysiert
Thiosalizylsäure	Karbonylverbindungen	Senning und Lawesson [21, 22]	Nur beschrieben für Aldehyde Reaktionsmedium Katalysator a) Eisessig Chlorwasserstoff b) Eisessig — c) Benzol <i>p</i> -Toluolsulfonsäure
<i>o</i> -prim- oder sek-Alkylmerkaptobenzoessäure	Chlor	Senning und Lawesson [20]	Nur beschrieben für die Synthese von 3,3-Dichlor-4-thiaisochroman-1-on
Thiosalizylsäure	Reaktive gem-Dihalogenide	Kaufmann und Rossbach [13]	Nur beschrieben für Phthalylchlorid

Die Thiosalizylsäure wurde von der Firma Schuchardt, München bezogen und schmolz zwischen 161° und 164° (Literatur: F: 164°).

Die Phthalaldehydsäure (F: 95°–97°, Literatur: F: 97°) war eine Sachspende der Firma Aldrich, Milwaukee (Dr. Bader), der wir hiermit unseren besten Dank aussprechen.

Versuchsanordnung

In einem 1-l-Kolben, der mit einem Rührer und einem Veresterungsaufsatz versehen war, wurde ein Gemisch von 0,10 Mol Thiosalizylsäure, 0,12 Mol Aldehyd, 1 g *p*-Toluolsulfonsäure und 500 ml Benzol etwa 3 Stunden am Rückfluss gekocht,

Tabelle 2. Ausbeuteverhältnisse bei der Reaktion

Thiosalizylsäure (I) + Benzaldehyd (II) $\rightarrow$ 3-Phenyl-4-thiaisochroman-1-on (III)

Reaktionsmedium	Verhältnis I:II	Katalysator	Wasserentziehendes Mittel	Ausbeute in %
Eisessig	1:1	Chlorwasserstoff	—	24 (Merkaptalsäure 69)
Eisessig	1:1	—	—	45
Benzol	1:1	—	Azeotrope Destillation	0
Benzol	1:1,2	<i>p</i> -Toluolsulfonsäure	Azeotrope Destillation	89
Alkohol	1:2	—	—	0
Äther	1:1	—	Wasserfreies Natriumazetat	0

wobei sich das gebildete Wasser im Veresterungsaufsatz ansammelte. Nach beendigter Umsetzung wurde die erkaltete Lösung filtriert und nach Zusatz von etwa 250 ml Äther so lange mit verdünnter Sodalösung ausgeschüttelt, bis die alkalische Reaktion der wässrigen Phase bestehen blieb. Anschliessend wurde die organische Phase mit Wasser neutral gewaschen, getrocknet und wie üblich aufgearbeitet.

3-(2-Furyl)-4-thiaisochroman-1-on

Ausgangsmaterial: 15,4 g (0,10 Mol) Thiosalizylsäure und 11,5 g (0,12 Mol) frisch destilliertes Furfurol. Nach einem Vorlauf von Furfurol gingen zwischen 160°/0,1 mm und 166°/0,1 mm 2,7 g = 12 % der Theorie über (F: 54°–57°). Nach mehrmaligem Umkristallisieren aus Äther und leichtsiedendem Petroläther schmolz das 3-(2-Furyl)-4-thiaisochroman-1-on (feine farblose Nadeln) zwischen 56,5° und 57,5°.

$C_{12}H_8O_3S$	gef.	C 62,11 %;	H 3,51 %;	S 13,69 %
	ber.	C 62,06 %;	H 3,47 %;	S 13,81 %

3-(2-Thienyl)-4-thiaisochroman-1-on

Ausgangsmaterial: 15,4 g (0,10 Mol) Thiosalizylsäure und 13,5 g (0,12 Mol) frisch destillierter 2-Thiophenalddehyd ($n_D^{20} = 1,5917$; Literatur: $n_D^{20} = 1,5920$). Rohausbeute 17,6 g = 71 % der Theorie (F: 76°–81°; Literatur [21]: F: 87°–88°). Nach mehrmaligem Umkristallisieren aus Äther schmolz das Produkt zwischen 87° und 88°.

3-(4-Chlorphenyl)-4-thiaisochroman-1-on

Ausgangsmaterial: 15,4 g (0,10 Mol) Thiosalizylsäure und 16,9 g (0,12 Mol) *p*-Chlorbenzaldehyd (F: 45°–48°; Literatur: F: 47,5°; Firma Kahlbaum). Rohausbeute 12,9 g = 47 % der Theorie (F: 111°–115°; Literatur [21]: F: 116°–117°). Nach mehrmaligem Umkristallisieren aus Äther schmolz das Produkt zwischen 116° und 117°.

3-(2-Nitrophenyl)-4-thiaisochroman-1-on

Ausgangsmaterial: 15,4 g (0,10 Mol) Thiosalizylsäure und 18,1 g (0,12 Mol) *o*-Nitrobenzaldehyd (F: 44°; Literatur: F: 44°; Firma Merck). Rohausbeute 5,8 g = 20 % der Theorie (F: 171°–175°; Literatur [21]: F: 177°–179°). Nach mehrmaligem Umkristallisieren aus Essigester schmolz das Produkt zwischen 177° und 179°.

3-(3-Methoxy-4-hydroxyphenyl)-4-thiaisochroman-1-on

Ausgangsmaterial: 15,4 g (0,10 Mol) Thiosalizylsäure und 18,6 g (0,12 Mol) Vanillin (F: 72°–81°; Literatur: F: 81°–82°). Aus der mit Natriumbikarbonatlösung extrahierten organischen Phase wurden 20,9 g Rohprodukt (73 % der Theorie) vom Schmelzpunkt 125°–135° erhalten. Nach mehrmaligem Umkristallisieren aus Alkohol schmolz das 3-(3-Methoxy-4-hydroxyphenyl)-4-thiaisochroman-1-on (farblose derbe rechtwinklige Prismen) zwischen 139° und 141°.

$C_{15}H_{12}O_4S$	gef.	C 62,51 %;	H 4,24 %;	S 11,04 %
	ber.	C 62,49 %;	H 4,20 %;	S 11,12 %

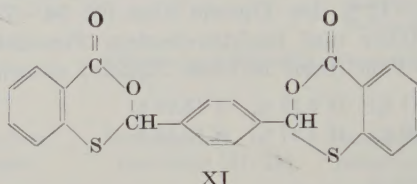
3-(4-*N,N*-Dimethylaminophenyl)-4-thiaisochroman-1-on

Ausgangsmaterial: 15,4 g (0,10 Mol) Thiosalizylsäure und 17,9 g (0,12 Mol) *p-N,N*-Dimethylaminobenzaldehyd (F: 69°–74°; Literatur: F: 74°). Die Umsetzung wurde mit 25,8 g (0,15 Mol) *p*-Toluolsulfonsäure durchgeführt. In Gegenwart von nur 1 g *p*-

Toluolsulfonsäure betrug die Ausbeute 4 % der Theorie. Die Benzollösung wurde samt Niederschlag ohne vorheriges Filtrieren aufgearbeitet. Es wurden 1,9 g Produkt (7 % der Theorie) vom Schmelzpunkt 169°–170° erhalten. Die analysenreine Substanz (farblose derbe rechteckige Tafeln) schmolz ebenfalls zwischen 169° und 170°.

$C_{16}H_{15}NO_2S$	gef.	N 4,90 %;	S 11,24 %
	ber.	N 4,91 %;	S 11,24 %

p-Bis-(4-thiaisochroman-1-on-3-yl)-benzol (XI)



Ausgangsmaterial: 30,8 g (0,20 Mol) Thiosalizylsäure und 13,4 g (0,10 Mol) Terephthalaldehyd (F: 113°–115°; Literatur: F: 116°; Firma Kahlbaum). Das äusserst schwerlösliche Produkt fiel während der Umsetzung aus, wurde abfiltriert und mit verdünnter Sodalösung digeriert. Rohausbeute: 38,5 g = 95 % der Theorie. F: 265°–275°. Nach zweimaligem Extrahieren mit Azeton im Soxhletapparat schmolz die Substanz (farblose derbe Nadeln) zwischen 272° und 273°. Da die Verbindung zwei asymmetrische Kohlenstoffatome besitzt, ist das Auftreten einer meso- und einer racem-Form zu erwarten. Auf Grund der ungünstigen Löslichkeitsverhältnisse konnten wir nur eine Form isolieren. Wir haben nicht untersucht, ob es sich um das Razemat oder um die meso-Form handelt.

$C_{22}H_{14}O_4S_2$	gef.	C 64,92 %;	H 3,55 %;	S 15,67 %
	ber.	C 65,01 %;	H 3,47 %;	S 15,78 %

3-(2-Karboxyphenylmerkpto)-phthalid

Ausgangsmaterial: 15,4 g (0,10 Mol) Thiosalizylsäure und 18,0 g (0,12 Mol) Phthalaldehydsäure. Rohausbeute 27,5 g = 96 % der Theorie (F: 228°–233°). Nach mehrmaligem Umkristallisieren aus Alkohol schmolz die Substanz (farblose feine Nadeln) bei 237°.

$C_{15}H_{10}O_4S$	gef.	C 63,09 %;	H 3,59 %;	S 11,19 %
	ber.	C 62,93 %;	H 3,52 %;	S 11,20 %

Abbau mit Raney-Nickel:

2 g 3-(2-Karboxyphenylmerkpto)-phthalid wurden unter Rühren 6 Stunden mit 150 ml *n*-Butanol und ca. 30 g frisch制备tem Raney-Nickel gekocht. Anschliessend wurde heiss vom Raney-Nickel abfiltriert, mit Äther aufgenommen und mit verdünnter Sodalösung ausgeschüttelt.

Aus der Ätherphase wurde nach Eindunsten im Rotationsevaporator eine neutrale Substanz vom Schmelzpunkt 65°–71° gewonnen. Nach Umkristallisieren aus Alkohol und Wasser schmolz die Substanz zwischen 69° und 73° (Mischschmelzpunkt mit authentischem Phthalid unverändert 69°–73°). Das Infrarotspektrum war identisch mit dem von authentischem Phthalid.

Die alkalische wässrige Phase wurde angesäuert und mit Äther ausgeschüttelt. Im Rotationselevator hinterblieb ein unscharf schmelzendes saures Produkt, das nach Reinigung durch Sublimation zwischen 119° und 121° schmolz (Mischschmelzpunkt mit authentischer Benzoesäure 119°–121°). Das Infrarotspektrum war identisch mit dem authentischer Benzoesäure.

ZUSAMMENFASSUNG

Die Methoden zur Darstellung von 4-Thiaisochroman-1-on-derivaten werden diskutiert. Vier neue 4-Thiaisochroman-1-on-derivate und ein neues Phthalidderivat werden beschrieben.

Wir sind dem Institutsvorstand, Herrn Professor A. Fredga, für die Überlassung des Arbeitsplatzes und *Jordbrukets Forskningsråd* für die finanzielle Unterstützung unserer Arbeit zu Dank verpflichtet.

Laboratorium für Organische Chemie, Chemisches Institut der Universität Uppsala, Uppsala, Schweden.

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Uppsala 1961. Almqvist & Wiksells Boktryckeri AB

Gamma-Globuline als Wirkstoff-Komponenten im normalen und im cancerösen Blutplasma¹

Von HANS VON EULER, HANS HASSELQUIST und ALF LARSEN

Mit 2 Figuren im Text

Globuline sollen sich, älteren Angaben zufolge von den Albuminen durch ihre Schwerlöslichkeit in salzfreiem Wasser unterscheiden. Bzgl. der γ -Globuline, siehe unten. Ihre langen Moleküle bestehen nicht nur aus aneinander gereihten Aminosäuren bzw. Peptiden, sondern sie enthalten auch andere Glieder, besonders Zuckerarten. Durch Ultracentrifugierung und Elektrophorese konnte die Proteine des Blutplasmas weiterhin getrennt werden, und Tiselius [1] hat die Globulin-Fraktion in α -, β - und γ -Globuline zerlegt. Durch fortgesetzte Elektrophorese und auch durch fraktionierte Salzfällung (Cohn [2]) lassen sich die Bestandteile des Plasmas weiter differenzieren und reinigen. Porter [3] gewann aus Kaninchen- γ -Globulin 3 Fraktionen, von denen eine (mit III bezeichnet) zur Krystallisation gebracht werden konnte.

Über den Mechanismus der γ -Globulin-Reaktion und über die Globuline als Abwehrstoffe ist noch wenig bekannt. Man weiss, dass die γ -Globuline Antikörper und also Träger einer Immunität bilden. Besonders bemerkenswert ist in diesem Zusammenhang das Properdin [4].

Eine vollständige Aufklärung der biologischen γ -Globulin-Wirkung setzt einen tieferen Einblick in den chemischen Bau dieser Stoffklasse und in die Natur ihrer reagierenden Gruppen voraus.

Die im Folgenden beschriebenen Reaktionen sind ausgeführt mit einem humanen γ -Globulin-Präparat Kb, das wir der Chemischen Fabrik Kabi, Stockholm, verdanken. Das Präparat Kb besteht aus einer einheitlichen kältegetrockneten γ -Globulin-Komponente.

Die physikalischen Eigenschaften der gelösten Plasma-Globuline findet man in einer Tabelle von Cohn [2, S. 127] zusammengestellt.

Wasserlöslichkeit des γ -Globulines Kb bei variierendem pH

Die in der Literatur für Globuline angegebene Schwerlöslichkeit in salzfreiem Wasser trifft *nicht* allgemein zu. (In verdünnter Kochsalzlösung sind Globuline leicht löslich.)

Die 1-%ige wässrige Lösung unseres γ -Globulin-Präparates Kb wird bei pH 4

¹ Herrn Professor Dr. Hermann Finck zum 60. Geburtstag gewidmet.

noch nicht gefällt. Bei diesem pH ist auch die Viskosität gegen diejenige bei pH 6 nur unbedeutend verändert.

Wir finden bei pH 7 30 mg γ -Globulin Kb in 0,1 ml reinen Wassers vollständig löslich.

Bei pH 6 sind 15 mg γ -Globulin in 0,1 ml Wasser fast löslich. Wurde die gleiche Lösung 30 Minute auf 55° erhitzt, so wurde keine vermehrte Fällung beobachtet. Eine merkbare Hitzedenaturation trat also bei dieser Temperatur nicht ein.

In einer Lösung von 15 mg γ -Globulin/ml Wasser entstanden kräftige Fällungen durch Zusatz von 0,015 ml HCl, bei pH 1,3.

Trichloressigsäure, als kräftiges Eiweissfällungsmittel bekannt, zeigt starke fällende Wirkung auf γ -Globulin schon bei pH 4.

Mit Platinchlorwasserstoffsäure bildet γ -Globulin beim Erwärmen auf etwa 50° eine gelbliche voluminöse Fällung.

Eine 1-prozentige Quecksilberchloridlösung gibt mit einer Lösung von 1,5 g γ -Globulin in 100 ml Wasser sofort einen gelblichen gelatinösen Niederschlag, der Hg enthält.

Papierelektrophorese der mit Formaldehyd bzw. mit Nitrit denaturierten γ -Globuline Kb

Die Einwirkung des Formaldehyds auf die Proteine des Rinderserums wurde zuerst von Guterfreund und Ogston [5] beschrieben. Eine entsprechende elektrophoretische Untersuchung an menschlichem Serum führten Tolmasquim *et al.* [6].

Terminale Aminosäuren menschlicher und tierischer Seren studierten Woo-Pok Lay und Polglase [7].

Probemenge zur Elektrophorese: 0,01 ml. Pufferlösung zur Elektrophorese: Veronal pH 8,6. Ionenstärke: 0,1. Papier: Schleicher & Schüll 2043B. Spannung: 100 Volt. Stromstärke: 1 mA. Zeit: 4 Stunden.

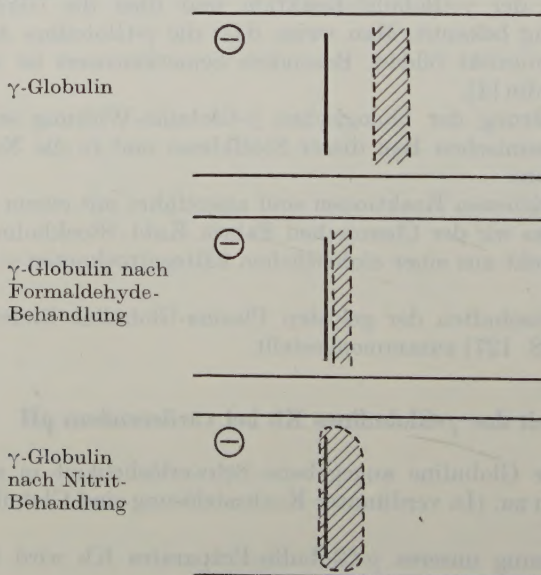


Fig. 1.

γ -Globulin nach Formaldehyd-Behandlung: 15 mg γ -Globulin, gelöst in 0,5 ml Wasser stand mit 0,1 ml 40 %iger Formaldehydlös. 60 Minuten bei 20°.

Nitrit-Versuch: 15 mg γ -Globulin gelöst in 0,5 ml Wasser. 30 mg, γ -Globulin in 1 ml Wasser + 0,1 ml Ac versetzt mit 0,1 ml Nitritlös. (enthaltend 10 mg NaNO_2/ml) stand 30 Min bei 10°, dann eingestellt mit Natronlauge auf pH 8,6 und mit Wasser auf 1,5 ml.

O-Versuch: 30 mg γ -Globulin in 1 ml Wasser + Natronlauge bis H 8,6. Volumen eingestellt auf 1,5 ml.

Die Ergebnisse sind aus den Fig. 1 ersichtlich.

Papierelektrophorese von γ -Globulin Kb, Rattenblutserum, Extrakt von Jensensarkom und Rattenmuskel

Pufferlösung für Extraktion und Elektrophorese: Veronal-acetat Puffer pH 8,6. Ionenstärke 0,1. Extraktmenge zur Elektrophorese: 0,01 ml. Spannung: 100 Volt. Stromstärke: 1 mA. Zeit: 2 Stunden.

Entwickler: Amidischwarz 10B (nach W. Grassmann u. a.). Gesättigte Lösung des Farbstoffs in Methanol enth. Eisessig zu 10 %.

Ergebnisse

Chromatogramm A. γ -Globulin Kb: ein kleiner Anteil verbleibt auf der Startlinie; die Hauptmenge wandert zur Anode.

Chromatogramm B. Das Serum (Rattenblut) wird hier in 4 Hauptkomponenten zerlegt, darunter Albumin. Das γ -Globulin befindet sich hier noch auf der Startlinie und zwar in sehr schwacher Konzentration.

Chromatogramm C. Extrakt einer cancerösen (Jensensarkom) Rattenmuskel (siehe [8]): keine Aufteilung in Zonen; ein Band breitet sich aus vom Startpunkt zur Anode.

Chromatogramm D. Extrakt eines Jensensarkoms; ein Band, breiter als bei Chromatogramm C, erstreckt sich zur Anode hin.

Versuche zur Endgruppen-Bestimmung an γ -Globulin

a. Umsetzung mit 2,4-Dinitrofluorobenzol (DNP) (F. Sanger)

200 mg γ -Globulin in 5 ml Wasser gelöst wurden versetzt mit 5 ml einer gesättigten NaHCO_3 -Lösung von 2,4 Dinitrofluorobenzol in 1 ml 95%igem Äthanol. Nach 3-stündigem Schütteln stand die Mischung über Nacht bei Zimmertemperatur und wurde dann mit Äther extrahiert bis Gelbfärbungen nicht mehr eintrat. Der in der wässrigen Schicht vorhandene gelbe Niederschlag von DNP- γ -Globulin betrug 135 mg. Das Produkt ist in allen üblichen Lösungsmitteln unlöslich [9].

b. Spaltung des DNP- γ -Globulins mit HCl

100 mg des DNP- γ -Globulins wurden mit 10 ml 6 N HCl im Bombenrohr bei 100° 18 Stunden erhitzt; die Lösung wurde filtriert und das Filtrat 3mal mit Äther extrahiert. Die wässrige Schicht, die nun die aus dem DNP- γ -Globulin abgespaltene Aminosäure sowie freie Aminosäuren enthielt, wurde papierchromatographiert.

Zum Vergleich wurde nun 15 mg des (unbehandelten) γ -Globulins mit 1,5 ml 6-N HCl im Bombenrohr bei 100° erhitzt und das aus diesem Kontroll-Versuch gewonnene Filtrat wurde ebenfalls chromatographiert.

c. Zweidimensionale Papierchromatographie

Papier: Whatman Nr. 1. Entwickler: Ninhydrin.

Richtung I: *n*-Butanol/Eisessig/Wasser 4:1:1.

Richtung II: Pyridin/Eisessig/Wasser 10:7:3.

In dem Chromatogramm des mit HCl gespalteten γ -Globulins tritt ein sehr deutlicher Flecken auf, dessen R_f betrug

R_f , Richtung 1 = 0,07,

R_f , Richtung 2 = 0,37.

Dieser Flecken *findet sich nicht* im Hydrolysat des DNP- γ -Globulins.

Die dadurch hervortretende Aminosäure betrachteten wir als die Endgruppe der Polypeptidkette und ist somit gebunden als DNP-Aminosäure im zweiten Chromatogramm.

Um zu ermitteln, welche Aminosäure im zweidimensionalen Chromatogramm des hydrolysierten DNP- γ -Globulins fehlte und dessen R_f -Werte im zweidimensionalen Chromatogramm des hydrolysierten γ -Globulins in der Richtung I 0,07 und in der Richtung II 0,37 betrugen, wurde zuerst ein eindimensionales Chromatogramm des hydrolysierten γ -Globulins verglichen mit freien Aminosäuren von entsprechendem R_f -Werten.

Eindimensional

I. Substanzen: Arginin, Histidin, Histamin, Taurin, Asparagin, Lysin und hydrolysiertes γ -Globulin.

Papier: Whatman nr 1.

Lösungsmittel: *n*-Butanol/Eisessig/Wasser 4:1:1.

Richtung der Chromatographie: Nach unten.

Zeit: 24 Stunden. Entwickler: Ninhydrin.

Die aktuelle Aminosäure des γ -Globulins ergab im oben beschriebenen Chromatogramm den R_f -Wert 0,05. Vergleichbar damit waren die Aminosäuren mit R_f = 0,05–0,06 nämlich Arginin, Histidin und Lysin.

Die DNP-Verbindungen dieser Aminosäuren wurden nun gleichzeitig mit dem hydrolysierten DNP- γ -Globulin chromatographisch untersucht.

II. *Chromatogramm* Fig. 2. Substanzen: DNP-Arginin, DNP-Histidin, DNP-Lysin, Hydrolysat des DNP- γ -Globulins, DNP-OH sowie DNP-NH₂. Chromatographische Lösungsmittel: 0,5-*M* Na₂HPO₄/0,5-*M* KH₂PO₄ 1:4. Richtung: Aufsteigend. Beobachtung: Visuell oder im UV.

Ergebnis: Nur DNP-Arginin erwies sich als identisch mit der DNP-Aminosäure im Hydrolysat des DNP- γ -Globulins.

Eine weitere Kontrolle wurde erhalten durch das folgende Chromatogramm:

III. *Chromatogramm.* Substanzen: DNP- γ -Globulin und DNP-Arginin. Papier: Whatman 1. Chromatographische Lösungsmittel: *n*-Butanol/5 %iges NH₃-Wasser 10:1. Richtung: Aufsteigend. Beobachtung: Wie im gleichen Chromatogramm.

Umsetzung von MZ-Chlorid mit Nikotinsäure

0,5 mMol (62 mg) wurden in 0,5 ml Wasser + 2 ml Dioxan suspendiert und unter Zusatz von 5 mMol (200 mg) MgO bei Zimmertemperatur 15 Min. verrührt. Nach weiterem Zusatz von 0,53 mMol (160 mg) MZ-Chlorid wurde 1 Stunde bei 20° umgeschüttelt. Nach Abschluss der Reaktionszeit wurde mit 1-*N* HCl bis pH 4 angesäuert. Die gelbe Fällung wurde abfiltriert und mit Wasser gewaschen. Roh-

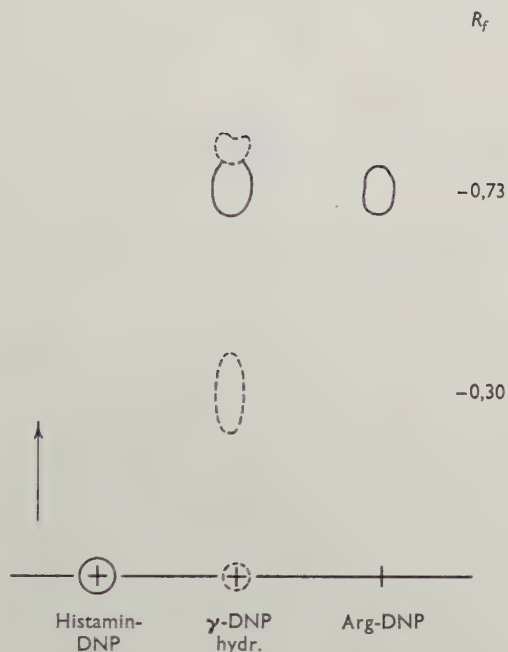


Fig. 2.

produkt: 85 mg vom Schmelzpunkt 100–105°. Aus Methanol-Wasser umkrystallisiert krystallisierte die Substanz in kleinen gelbbraunen Prismen vom Umwandlungspunkt 109–112°. Ein Mischschmelzpunkt mit dem aus Nikotinsäureamid-MZ-Produkt zeigte dass die beiden Substanzen identisch sind. Dass die entstandene Verbindung tertiären Stickstoff enthält wurde nach Ohkuma [10] geprüft.

Fortsetzung in folgender Mitteilung.

Stockholm, Universität, Vitamin-Institut.

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Removal of contaminating plasminogen from purified bovine fibrinogen

By KURT BERGSTRÖM and PER WALLÉN

With 5 figures in the text

Introduction

Although several methods have been suggested for the purification of bovine fibrinogen, it has been shown that even highly purified preparations contain traces of plasminogen (Blombäck and Blombäck, 1956; Wallén and Bergström, 1957, 1958). This despite the fact that all but traces of protein impurities have been removed from the fibrinogen.

The importance of such contamination when fibrin and fibrinogen are used as substrates in determination of different components of the fibrinolytic system has been stressed earlier (Lassen, 1952; Müllertz and Lassen, 1953; Sherry, Fletcher and Alkjærsig, 1959). Thus, the presence of plasminogen makes preparations of bovine fibrinogen or fibrin useless for estimation of plasmin in solutions containing plasminogen activators. In such systems, the rate of digestion is dependent on the concentration of activator. A fibrin from which the contaminating plasminogen has been removed should, however, constitute an adequate substrate for the fibrinolytic determination of plasmin.

Plasminogen seems to have a marked tendency to form complexes with other proteins (Remmert and Cohen, 1949). This phenomenon has hampered purification of the proenzyme and may be responsible for the difficulties involved in preparing fibrinogen devoid of plasminogen.

It has recently been shown that the solubility of some pure plasminogen preparations in neutral buffers is increased if small amounts of ϵ -aminocaproic acid or lysine are added to the solvents (Alkjærsig, Fletcher and Sherry, 1959 a; Hagan, Ablondi and De Renzo, 1960). We have devised a simple method for the preparation of highly purified human plasminogen, in which this substance is adsorbed on DEAE cellulose, and eluted by means of buffers containing low concentrations of lysine (Wallén and Bergström, 1959, 1960). It is evident that lysine is able to interact with the plasminogen, bringing about a dissociation of its complexes with other proteins.

The purpose of this paper is to present a method for the removal of contaminating plasminogen from purified bovine fibrinogen, based on this effect of lysine.

Material and methods

Bovine fibrinogen, fraction I-4, was prepared according to Blombäck and Blombäck (1956). This preparation has a coagulability of 98–100 per cent.

Urokinase. Ploug and Kjeldgaard (1957) have devised a method for the purification of urokinase in which the first step is an adsorption from urine on a column of silica gel. The method used here is based on a batch-wise adsorption of urokinase on Hyflo Super-Cel. Normal male urine was collected with addition of tricesol, and the material worked up within 20 hours. 1000 g of Hyflo Super-Cel¹ were suspended in 100 l of urine. The Hyflo was then collected on a large Büchner funnel, washed several times with 0.15 *M* NaCl and then with distilled water. The adsorbed urokinase was eluted from the Hyflo with 5000 ml of 1 *M* ammonium hydroxide. After centrifugation of this eluate, a partly purified urokinase was obtained by adjusting the pH of the eluate to 1.5 with HCl, and saturating with NaCl. After centrifugation, the partly floating, partly sedimented precipitate was dissolved in a small quantity of water, dialyzed against distilled water, and freeze-dried. The resulting brownish powder weighed from 1.5 to 2 g.

Two g of such lyophilized material were dissolved in 600 ml of distilled water whereupon 200 ml of 2 *M* ammonium hydroxide were added under gentle stirring at 0°C. 80 g of ammonium sulphate were added, and the precipitate obtained was removed by centrifugation. After adjusting the pH of the supernatant to 7, the urokinase was precipitated by adding 450 g of ammonium sulphate. The precipitate was suspended in and dialyzed against distilled water at 0°C, and then freeze-dried. The weight of this greyish-white powder usually ranged from 500 to 800 mg. The yield of activity was about 80 per cent as compared to that of the urine.

Casein. Hammarsten casein was treated as described by Müllertz (1955).

Glycine. Glykokoll Erg. B6 (Badische Anilin- & Soda-Fabrik A.G.).

Lysine. L-lysine monohydrochloride from Hopkin-Williams and from California Corp. (B-grade reagent).

Thrombin was prepared according to Seegers (Seegers, McClaughry and Fahey, 1950; Seegers, 1952) and kindly placed at our disposal by Dr S. Magnusson, Karolinska Institutet, Stockholm.

Determination of fibrinogen. Fibrinogen was determined as fibrin with the syneresis method of Morrison (1947), as modified by Blombäck and Blombäck (1958). After coagulation with thrombin, the clot was allowed to synerize on a silk cloth, and washed several times with 0.15 *M* NaCl. The fibrin was dissolved in alkaline urea solution, and the amount of protein calculated from the optical density at 282 m μ . The purity was expressed as coagulable protein in per cent of total protein.

Estimation of plasminogen content of fibrinogen. In the preparations to be tested there is a large excess of fibrinogen which will constitute part of the substrate. When comparing plasminogen activity in different preparations, it is therefore necessary to dilute them to the same fibrinogen concentration. The method used is based on the activation of the plasminogen with urokinase and the subsequent digestion at 37°C. The optical density at 275 m μ of the filtrate after precipitation with perchloric acid is a measure of the rate of proteolysis. An addition of casein to the digestion mixture has been found to increase the sensitivity of the method.

The following method has been adopted as a standard procedure for estimation of the amount of plasminogen present in various fibrinogen preparations. 10 ml of 2 per cent casein in 0.1 *M* sodium phosphate buffer, pH 7.3, was added to 10 ml of 2 per cent fibrinogen in 0.3 *M* NaCl. After prewarming in a water bath at 37°C, activation of the plasminogen was started by the addition of 5 ml of a 0.1 per cent solution of urokinase in distilled water. Two ml of the digestion mixture

¹ Supplied by Johns-Manville Corporation, New York.

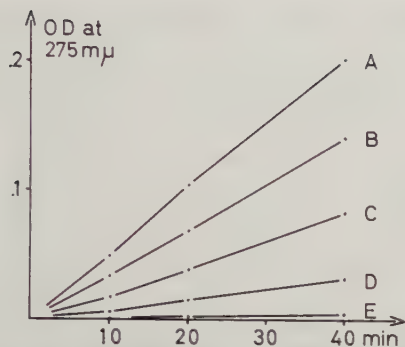


Fig. 1. Proteolytic activity appearing after addition of urokinase to mixtures of fraction I-4 and fraction I-4 L. *A* = 100 % fraction I-4, *B* = 75 % fraction I-4 + 25 % fraction I-4 L, *C* = 50 % fraction I-4 + 50 % fraction I-4 L, *D* = 25 % fraction I-4 + 75 % fraction I-4 L, *E* = 100 % fraction I-4 L.

were added to 3 ml of 1.7 *M* HClO₄, immediately after the addition of urokinase, and then at different times during incubation. The perchloric acid precipitates were allowed to stand at room temperature for at least 30 minutes, and were then removed by filtration through Munktell 00 filter papers. The optical density of the filtrates was measured at 275 mμ in a Beckman DU spectrophotometer against distilled water. The increase in optical density was plotted against incubation time. The slope of this curve is an approximate measure of the plasminogen content of the fibrinogen. This is shown in Fig. 1, where a plasminogen-containing fibrinogen (fraction I-4, *A*) has been mixed in various proportions with a fibrinogen from which the plasminogen had been removed (fraction I-4 L, *E*).

N-terminal and free amino acids in the fibrinogen preparations were determined by Edman's phenylisothiocyanate method, as applied by Blombäck and Yamashina (1958).

Experimental

Preparation of fibrinogen free from plasminogen activity

As starting material for the further purification of bovine fibrinogen we used fraction I-4, prepared as described by Blombäck and Blombäck (1956). This highly purified preparation is free from plasmin activity. However, plasmin activity can be detected after activation with urokinase (Fig. 1, *A*). The final step in preparation of this fraction is precipitation under the following conditions: ethanol 6.5 per cent, glycine 0.45 *M*, protein concentration about 0.15 per cent, pH about 7, and ionic strength about 0.08. Concentrated solutions of this fraction in 0.3 *M* NaCl were stored frozen at -15°C until used.

The purification was performed by reprecipitation of fraction I-4 with ethanol, in the presence of lysine monohydrochloride and glycine. The following procedure was adopted as being the most convenient.

An appropriate amount of fraction I-4 was dissolved in and diluted with 0.3 *M* NaCl to a protein concentration of about 1.8 per cent. Precipitation was then performed under the following conditions: ethanol 10.4 per cent, lysine monohydrochloride 0.20 *M*, glycine 0.20 *M*, protein concentration about 0.15 per cent, pH about 7, and ionic strength about 0.28. The procedure was as follows.

Table 1. Coagulability and yield of the dialyzed fibrinogen preparations.

Fraction I-4	Coagulability per cent		Yield in per cent of fraction I-4
	Fraction I-4	Fraction I-4 L	
97.7		99.6	65
97.7		100.3	90
96.2		99.7	93
96.8		99.4	83
96.2		98.8	82

To every 100 ml of diluted fibrinogen solution were added with stirring 480 ml of a mixture consisting of 72.5 ml of 2 *M* glycine, 72.5 ml of 2 *M* lysine HCl, 55 ml of distilled water and 280 ml of a phosphate buffer pH 7.3–7.5, ionic strength 0.1 (45 ml of 0.2 *M* KH_2PO_4 + 155 ml of 0.2 *M* K_2HPO_4 + distilled water to 1000 ml). After cooling to 0°C in a low-temperature thermostat tank, 137 ml of 53.3 per cent ethanol were added. During this addition the temperature was lowered to –4°C. After standing for 15 minutes at –4°C, the mixture was centrifuged for 5–10 minutes at 2000 *g*. The precipitate was dissolved in 100 ml of 0.3 *M* NaCl. This procedure was repeated twice. After the third precipitation, the fibrinogen was dissolved in 0.3 *M* NaCl to a protein concentration of approximately 2.5 per cent. The solution was dialyzed for about 48 hours against several changes of 0.3 *M* NaCl at 0°C. To provide a suitable sample of the starting material for comparative analyses, lysine was added to a small amount of it (about 1 mM of lysine/g of protein), whereupon this solution was dialyzed as stated above. The dialyzed solutions were stored frozen at –15 to –20°C.

Purified fibrinogen preparations prepared with the present procedure are denoted in the following as fraction I-4 L, to distinguish them from the starting material (I-4), treated as described above.

Analytic results

Coagulability and yields. The purity of the fibrinogen preparations was checked by determination of their coagulability. After dialysis at 0°C for a long time the coagulability of fraction I-4 has a tendency to decrease (Blombäck, 1958). This effect has not been observed as concerns fraction I-4 L, which even after extensive dialysis as a rule has a coagulability of 99 to 100 per cent (Table 1). The yield of fraction I-4 L was usually 80 to 95 per cent of the starting material (fraction I-4).

Determination of plasminogen content. The proteolytic activity in the fibrinogen preparations after activation with urokinase, was estimated on casein with the method outlined above. A strong proteolytic effect was present in fraction I-4 (Fig. 1, *A*), whereas a very weak activity could be detected in fraction I-4 L (Fig. 1, *E*).

In order to ascertain whether the weak proteolysis often observed in the I-4 L preparations could possibly depend on the proteolytic activity of urokinase, the aforementioned determination was repeated, but with different amounts of urokinase (Fig. 2). As can be seen from the figure, a decrease in urokinase concentration produced only a very slight reduction in the fibrinolytic activity of fraction I-4. The proteolytic activity in the I-4 L preparation was, however, approximately proportional to the concentration of urokinase. Practically no proteolytic activity was observed when the lowest urokinase concentration was used (Fig. 2, *F*).

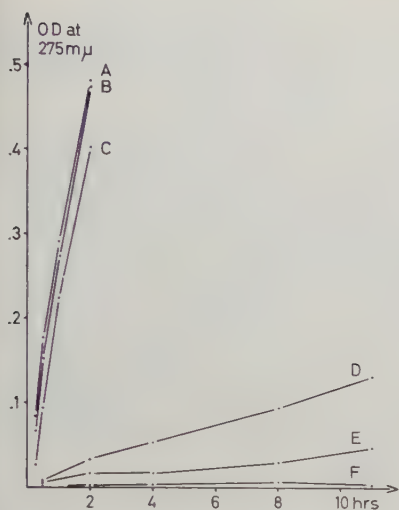


Fig. 2.

Fig. 2. Proteolytic activity of fraction I-4 (*A*, *B* and *C*) and fraction I-4 L (*D*, *E* and *F*) after addition of different amounts of urokinase. Concentration of urokinase solution: *A* and *D* = 6 mg/ml, *B* and *E* = 3 mg/ml, *C* and *F* = 0.15 mg/ml.

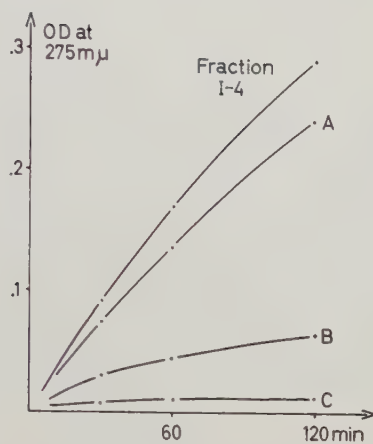


Fig. 3.

Fig. 3. Effect of lysine concentration on plasminogen content of reprecipitated fibrinogen. *A* = 0.02 *M* lysine + 0.38 *M* glycine, *B* = 0.08 *M* lysine + 0.32 *M* glycine, *C* = 0.20 *M* lysine + 0.20 *M* glycine. Fraction I-4 = starting material.

The stability of fibrin clots in the presence of urokinase was tested as follows. In small test tubes the following reagents were mixed: (1) varying amounts of urokinase dissolved in 0.5 ml of 0.05 *M* sodium phosphate buffer, pH 7.3, (2) 0.4 ml of a 0.5 per cent solution of fibrinogen in 0.15 *M* NaCl, and (3) 0.1 ml of a thrombin solution in 0.15 *M* NaCl containing about 30 NIH units/ml. The tubes were incubated in a water-bath at 37°C, and the time necessary for clot dissolution recorded. Table 2 shows a comparison between the stability of clots prepared from fraction I-4 and

Table 2. Comparison between the stability of fibrin clots prepared from fraction I-4 and from fraction I-4 L after addition of varying amounts of urokinase.

Urokinase mg/ml	Lysis time	
	Fraction I-4, minutes	Fraction I-4 L, days
1.6	24	About $\frac{5}{8}$
0.8	19	> 6
0.4	16	> 6
0.08	20	> 6
0.04	29	> 6
0.008	68	> 6
0.004	About 120	> 6

from fraction I-4 L. All the clots prepared from fraction I-4 dissolved in the course of 16 to 120 minutes, whereas those prepared from the further purified fibrinogen were stable, except for the clot to which the largest amount of urokinase was added. The implications of these findings will be discussed in a later section.

N-terminal and free amino acids. In order to rule out the possibility that any lysine included in the protein could influence the analysis, e.g. by inhibiting activation of the plasminogen, the free and *N*-terminal amino acids in the lysine-treated, dialyzed fibrinogen (I-4 L) were determined by Edman's phenylisothiocyanate method. According to Blombäck and Yamashina (1958), glutamic acid and tyrosine are present as *N*-terminal amino acids in bovine fibrinogen, each in a concentration of 2 moles/mole of fibrinogen. We obtained a yield of 60 and 75 percent, respectively. In addition to these amino acids, traces of glycine were found. No lysine could be detected. By adding small amounts of lysine to the fibrinogen, we were able to convince ourselves that the quantity of this amino acid in the dialyzed preparations amounted to less than 10^{-6} moles/g of fibrinogen. It is therefore highly improbable that lysine can have had any effect on the analyses.

Discussion

Methods in which the substrate consists of fibrin or fibrinogen have been widely used for estimation of components of the plasminogen-plasmin system. Sensitive, rapid and simple methods have been developed. The usefulness of these substrates has, however, been hampered by the fact that all hitherto available fibrinogen preparations have been contaminated with plasminogen, which has made them sensitive to plasminogen activators as well as to plasmin.

To circumvent this drawback, attempts have been made to eliminate the plasminogen in preformed fibrin clots by denaturation, or elution of the plasminogen. Thus, Lassen (1952) modified the fibrin plates of Astrup and his co-workers by heating the plates to 85°C. Such fibrin plates, in which the plasminogen has been destroyed by heat, are not digested by plasminogen activators. They have been used extensively by Astrup and his group, in combination with unheated plates, to distinguish between activators and proteolytic enzymes in various tissues and body fluids. A disadvantage of this method is that heating reduces the sensitivity of the fibrin to plasmin (Alkjærsig, Fletcher and Sherry, 1959 b).

Alkjærsig, Fletcher and Sherry (1959 b) tried to produce plasminogen-deficient, ^{131}I -labelled human fibrin clots by washing them vigorously with phosphate saline buffer. Clots thus obtained are still sensitive to plasminogen activators, although the sensitivity is lowered.

Obviously, the method of choice for preparing clots resistant to lysis by plasminogen activators implies the use of a fibrinogen preparation devoid, or practically devoid, of plasminogen. With this object in view, Milstone (1941), Sherry (1954) and Markus and Ambrus (1960) reprecipitated fibrinogen several times with ammonium sulphate. Although these preparations are a definite improvement, they still seem to contain significant amounts of plasminogen, which may interfere with the analyses when solutions of low fibrinolytic activity are to be assayed.

Celander and Guest (1960) recently reported a method for the preparation of profibrinolysin-free fibrinogen, based on multiple adsorption of fibrinogen on calcium

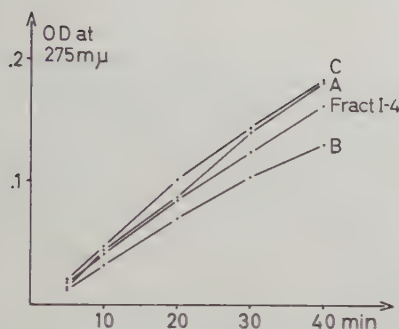


Fig. 4.

Fig. 4. Effect of ionic strength on plasminogen content of fibrinogen preparations reprecipitated three times without lysine. Ionic strength: $A = 0.08$, $B = 0.18$, $C = 0.28$. Fraction 1-4 = starting material.

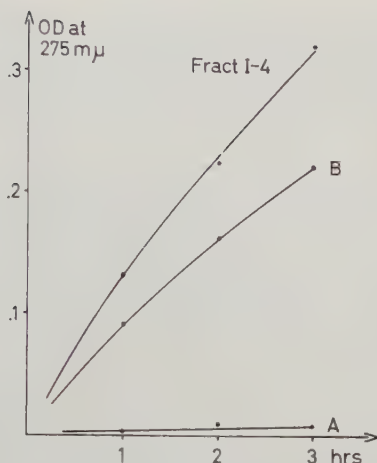


Fig. 5.

Fig. 5. Urokinase-induced proteolytic activity of fraction I-4 L before (A) and after (B) addition of the supernatant fraction. The proteolytic activity induced in the starting material (fraction I-4) is shown for comparison.

phosphate gel. They did not, however, give any detailed information about the method of preparation, nor about the analysis of their product.

The simple method for the removal of contaminating plasminogen from bovine fibrinogen described in the present paper is based on the specific action of lysine on plasminogen. We used a highly purified fibrinogen preparation (Blombäck & Blombäck, 1956) as the starting material, but less pure preparations (fraction I-2) can also be used with good results.

In order to obtain a fibrinogen free from plasminogen activity, the fibrinogen had to be precipitated at a high lysine concentration. When the concentration of lysine monohydrochloride was lower than $0.20 M$, the fibrinogen was still significantly contaminated with plasminogen (Fig. 3).

Three precipitations of the fibrinogen in the presence of lysine were required to remove the plasminogen. After one precipitation, or even two, traces of plasminogen could still be detected.

When the fibrinogen was precipitated at an ethanol concentration of 6.4 per cent, as in the original method for preparing fraction I-4, the yield of fibrinogen was often fairly low (about 60 per cent). An increase in the concentration of ethanol to 10.4 per cent considerably improved the yield (to 80–95 per cent). As far as could be seen from the analytic data, the higher concentration of ethanol did not impair the quality of the fibrinogen.

Precipitation of fraction I-4 L was performed at an ionic strength of 0.28, whereas that used in precipitation of the ordinary fraction I-4 is 0.08. To exclude the possibility that the greater purification was due to the increased ionic strength, a series of experiments was made in which the fibrinogen was precipitated from solutions of

varying ionic strength, and containing glycine in a concentration of 0.40 *M* as the sole amino acid. Thus, precipitation was carried out at an ionic strength of 0.08 (as in preparation of fraction I-4), 0.18 and 0.28, respectively. The strength was increased by adding sodium chloride to the buffer in which the fibrinogen was dissolved. In every other respect, the conditions were the same as in the method described above. It can be inferred from Fig. 4 that the plasminogen content of the fibrinogen precipitated at high ionic strength did not differ from that of the starting material (fraction I-4). That the removal of plasminogen from the fibrinogen in our procedure was not due to an increased ionic strength also appears from some experiments in which the lysine monohydrochloride was replaced by ϵ -aminocaproic acid and at which the ionic strength was 0.08. The fibrinogen thus obtained was also devoid of plasminogen activity. A difference in the properties of fraction I-4 L and fraction I-4 is that the solutions of fraction I-4 L are always more opalescent than the solutions of the starting material. By replacing lysine with ϵ -aminocaproic acid, however, we have obtained fibrinogen solutions which are more clear.

With a view to demonstrating that the stability of the fibrinogen in the presence of urokinase was, in fact, due to the removal of plasminogen, attempts were made to recover the plasminogen from the supernatant of fraction I-4 L. One-tenth of the combined supernatants from a preparation of fibrinogen purified by the present method was thoroughly dialyzed against distilled water and freeze-dried. This material was then combined with one-tenth of the purified fibrinogen. The proteolytic activity of the mixture, after activation with urokinase, corresponded to about 70 per cent of that present in the starting material (fraction I-4) when activated in the same way (Fig. 5).

One difficulty encountered in our work was that the activator used, urokinase, has a weak proteolytic activity, as demonstrated earlier by Ploug and Kjeldgaard (1957). This made it difficult to determine whether the weak proteolysis observed when urokinase was added to the further purified fibrinogen was, in fact, due to traces of plasminogen, or to the proteolytic activity of urokinase *per se*.

In the lysine-treated fibrinogen, the rate of proteolysis was approximately proportional to the concentration of urokinase, and was scarcely measurable in the lowest concentrations used (Fig. 2, *D*, *E* and *F*). In the starting material, on the contrary, the rate of proteolysis was almost as high with the lowest concentration of urokinase as with the highest (Fig. 2, *A*, *B* and *C*). These results could be expected if it is assumed that proteolysis was due mainly to the urokinase itself in the former case, and to the activation of plasminogen in the latter. Studies of the fibrinolytic activity in fibrin clots, after the addition of different amounts of urokinase, also revealed dissimilarities between the two types of fibrinogen preparations. Thus, in clots prepared from the further fractionated fibrinogen, fibrinolytic activity was observed only in those containing the largest amount of urokinase. The optimal fibrinolytic activity (16 minutes) of clots prepared from the starting material, was, on the contrary, observed at urokinase concentrations that were evidently incapable of lysing clots prepared from lysine-treated fibrinogen (more than 6 days required for lysis).

It has been claimed by Dalby and Mertz (1957) that sodium chloride has an inhibitory effect on the activation of bovine plasminogen whereas sodium citrate has not. The possibility existed that the high concentration of sodium chloride used in the determination of plasminogen had influenced our results. Therefore a fraction I-4 and a fraction I-4 L were divided, each into two halves, one of which was dis-

solved in and dialyzed against 0.05 *M* sodium citrate (ionic strength 0.3) whereas the other half was treated in the ordinary way. In neither of the two preparations did the replacement of sodium chloride with sodium citrate have any effect on the proteolytic activity obtained after activation with urokinase.

SUMMARY

A method for removal of the plasminogen from purified bovine fibrinogen is described. The method is based on the ability of lysine to increase selectively the solubility of plasminogen in the presence of other proteins. An account is given of the properties of the fibrinogen obtained, as disclosed by analyses of its coagulability, plasminogen content and *N*-terminal amino acids. As far as can be seen from these analyses this fibrinogen contains no plasminogen. It is possible to recover most of the plasminogen in the supernatant after precipitation of the fibrinogen.

ACKNOWLEDGEMENTS

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Spaltungen der Nukleinsäuren in saurer Lösung

Von HANS VON EULER, HANS HASSELQUIST und ALF LARSEN

Mit 2 Figuren im Text

Der dominierende Einfluss der Desoxyribo-Nukleinsäuren (DNS) auf den gesamten Stoffwechsel, besonders auf das Wachstum und auf die cellularen Vererbungsvorgänge kann auf die Mitwirkung der verschiedenen in den DNS-Ketten enthaltenen Nukleotidgruppen (Polynukleotid-Fractionen) zurückgeführt werden. Dass DNS irgendwie als Matrice beim Aufbau der Proteine dient, ist nunmehr kaum zu bezweifeln. Durch die Hypothese von Crick und Watson und durch die grundlegenden experimentellen Arbeiten von Chargaff und von Kornberg über den Bau der DNS sind wertvolle Ergebnisse gewonnen worden. Immerhin steht die DNS-Forschung hinsichtlich der Wirkungsphasen und Ergone, durch welche die Oligo- und Poly-Nukleotide an den Lebenserscheinungen teilnehmen, noch in den Anfängen.

Die Polynukleotidketten der DNS funktionieren als Matrizen in zweierlei Reaktionen:

- a) bei der DNS-Reproduktion, die unter Erhaltung der ursprünglichen Sequenz der 4 Basen eintritt,
- b) bei den mit dem DNS-Aufbau gekoppelten Protein-Synthesen (Kornberg, Nobelvorlesung 1959, S. 165), insofern als eine gewisse Peptid-Sequenz durch eine gewisse Nukleotid-Sequenz hervorgerufen wird.

Die enzymatische DNS-Spaltung ist von mehreren Gesichtspunkten aus wiederholt studiert worden. Die nicht-enzymatische DNS-Spaltung hat in diesem Institut A. Fonó [1] bearbeitet.

Zum Vergleich mit DNS sind im Folgenden einige mit Ribonukleinsäure (RNS) ausgeführten Spaltungsversuche angegeben. Zu ihrer Ergänzung erscheint es notwendig, den Einfluss von Säuren auf DNS und RNS bzw. den Übergang der beiden Arten von Nukleinsäuren in einander kennen zu lernen. Das Hauptziel unserer Arbeit ist die Isolierung und Identifizierung der aus DNS gewinnbaren *Oligo-Nukleotide*, die als Coenzyme funktionieren können.

Die aus DNS in saurer Lösung entstehenden Purin- und Pyrimidin-Basen

Spaltung

50 mg DNS-Präparat wurden in 25 ml Wasser gelöst und unter kräftigem Umrühren mit 25 ml 1-n HCl versetzt, wodurch DNS teilweise in feinsten Verteilung ausfällt. Unter fortgesetztem Rühren wurde die Flüssigkeit 5 min. auf 50° gehalten

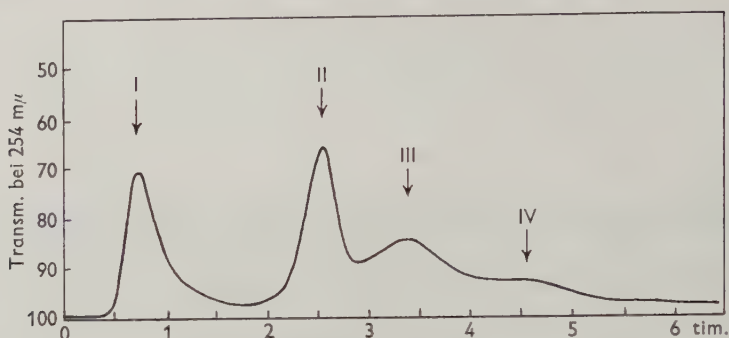


Fig. 1. Säule: Sephadex G 75; Apparat: LKB Uvicord + Schreiber + Fraktionssamler.

und dann schnell auf Raumtemperatur abgekühlt. Nach $\frac{1}{2}$ Stunde wurde vom ungelösten abfiltriert und das schwach braunrot gefärbte Filtrat auf einer Gelsäule (Sephadex G 75) chromatographiert (Figur 1).

Vorversuch

Die Gelsäule (8 × 300 mm) war mit Uvicord (LKB) als Detektor (Feste Wellenlänge 254 $m\mu$), Schreiber und Fraktionssamler verbunden. — Tropfgeschwindigkeit: 0,75 ml/10 min. Papiergeschwindigkeit des Schreibers: 2 cm/Stunde.

In einem Vorversuch mit 0,01 ml DNS und Elution durch Wasser wurden 4 deutlich getrennte Fraktionen einzeln im Vakuum auf etwa 0,5 ml eingengt und auf Phosphat sowie auf tertiären N (Purin und Pyrimidin) geprüft.

Fraktion nr	I	II	III	IV
Phosphatprobe	+	+	+	—
Tertiäres N	+	+	+	+

Um eine grössere Menge der Fraktionen I, II, III und IV zu erhalten, wurde nun eine Gelsäule (Sephadex G 75) mit den Dimensionen 13 × 270 mm zur Prüfung der Fraktionen angewandt. Elution durch Wasser.

Jede der 4 Fraktionen wurde nun einzeln im Vakuum auf etwa 0,5 ml eingengt, hierauf mit 3 ml 1-n HCl versetzt und sodann 20 Min. bei 60° erwärmt und weiter im Vakuum auf etwa 0,1 ml eingengt.

Papier-Circular-Chromatogramm 1 (Munktell 3)

Die Fraktionen I, II, III und IV.

Ferner die mit 1-n HCl gespaltenen Fraktionen SI, SII, SIII und SIV sowie Cytosin, Guanin und Adenin.

Lösungsmittel: Iso-propanol ÷ Wasser 7 : 3. Entwickler: $Hg(NO_3)_2/Na_2S$.

Resultat: Die Fraktionen I, II, III und IV verbleiben auf dem Start; ihr Molekulargewicht ist vermutlich hoch.

Cytosin	0,74
Adenin	0,80
Guanin	0,63
Fraktion SI	0,00
SII	0,66–0,80
SIII breite Linie	0,48–0,67
SIV Keine sichtbare Linie	

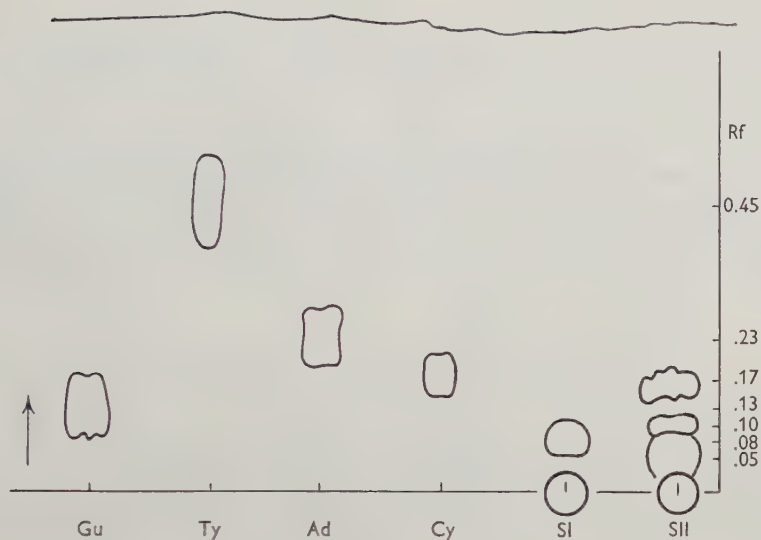


Fig. 2.

Die Fraktion II kann 2 Hauptlinien enthalten, die nach Spaltung mit 1-n HCl auftreten, und zwar die Linien von Adenin und Cytosin. Die gleiche Fraktion (II) enthält *vor* der Spaltung gebundenes Phosphat, wie aus den Vorversuch (S. 514) hervorgeht.

Da das Phosphat an Desoxyribose gebunden ist, muss auch diese Komponente in die ungespaltene Fraktion II eingehen.

Fraktion SIII enthält vielleicht Guanin; dieses Purinderivat findet sich auch in der Fraktion III (vor der HCl-Spaltung) und zwar als Phosphat und als Nukleotid.

Um mehr Versuchsmaterial zu erhalten wurde nun DNS mit 1-n HCl gespalten, wie im Vorversuch (S. 513), worauf die dabei erhaltene Lösung mit der grösseren Sephadex-Säule (siehe S. 514) chromatographiert wurde.

Die so erhaltenen 4 Fraktionen I, II, III und IV wurden folgendermassen getestet:

1. Mit Feulgen-Reagens: a) unmittelbar, b) nach 20 Min. Reaktionszeit,
2. durch die Metallionen von Zn, Co, Mg und Al bei pH 7.5 unter KAc-Zusatz.

Fraktion nr.	Feulgen a)	Feulgen b)	Metallionen 2
I	—	+	—
II	—	+	—
III	—	—	—
IV	—	—	—
0-Probe	—	—	—

Vgl. die Tests im Vorversuch (S. 514).

Das Chromatogramm (S. 514) (Iso-propanol:Wasser, 7:3) zeigt

SI (gespaltenes I) wandert nicht.

SII kann Adenin und Cytosin enthalten.

SIII kann Guanin enthalten.

SIV liefert keinen Flecken.

Eine definitive Entscheidung über das Vorkommen der Komponenten in den untersuchten Fraktionen wurde gesucht durch

Chromatogramm 2 (aufsteigend)

Papier: Whatman nr. 1. Lösungsmittel: Wasser-ges. *n*-Butanol. Entwickler: $\text{Hg}(\text{NO}_3)_2/\text{Na}_2\text{S}$.

Getestete Substanzen: Adenin, Cytosin, Guanin, Fraktionen SI, SII, SIII und SIV.

Ergebnis:	Adenin	$R_f = 0,33$	Cytosin	$R_f = 0,13$
	Guanin	0,00	SII	$R_{f1} = 0,00$
	SI	0,00		$R_{f2} = 0,13$
	SIII	0,00		
	SIV Wegen zu kleiner Substanzkonzentration nicht bestimmbar.			

Chromatogramm 3

Papier, Entwickler und Substanzen wie in Chromatogramm 2.

Lösungsmittel: *n*-Butanol-5%ige Ammoniaklös. 84:14.

Ergebnis:	Adenin	$R_f = 0,28$	Cytosin	$R_f = 0,16$
	Guanin	0,00	SI	0,00
	SII	$R_{f1} = 0,00$	SIII	0,00
		$R_{f2} = 0,15$		
	SIV liess sich nicht entwickeln.			

Aus den obigen Chromatogrammen 1-3 lassen sich folgende Schlüsse ziehen:

1. — Da nach Chromatogramm 1 Adenin, Cytosin und Guanin wandern dagegen nicht SI, kann Fraktion SI diese Basen nicht enthalten.
2. — Nach Chromatogramm 1 kann die eine Komponente von SII identisch sein mit Adenin oder Cytosin. Nach Chromatogramm 2 und 3 kann nur Cytosin mit der Komponente in Fraktion SII identisch sein.
3. — Nach Chromatogramm 1 kann Fraktion SIII mit Guanin identisch sein. Die gleiche Tatsache zeigte sich durch die Chromatogramme 2 und 3, nach denen SIII und Guanin auf der Startlinie verbleiben.
4. — SII enthält anscheinend 2 Substanzen, von welchen die eine, Cytosin von besonderem Interesse ist, da SII durch Hydrolyse der Ausgangsfraktion II (siehe S. 514) erhalten wurde und somit aus einem Dinukleotid bestehen kann.

Eine zweite Komponente von SII kann möglicherweise eine andere Base enthalten als Cytosin, eventuell Thymin, das in den obigen Chromatogrammen nicht untersucht wurde. Die Hauptfraktion II würde demnach aus einem Dinukleotid bestehen. Um hierüber Aufschluss zu gewinnen, wurde ein Chromatogramm ausgeführt, das u. a. Thymin als Vergleichs-Substanz enthielt.

Chromatogramm 4 (aufsteigend)

Papier: Whatman nr. 1. Lösungsmittel: *N*-Butanol-Ameisensäure-Wasser, 77:10:13.

Substanzen: Adenin, Guanin, Cytosin, Thymin, ferner die Fraktionen SI, SII, SIII und SIV. (Siehe Fig. 1.)

Ergebnis. Die Fraktion SI. mit obigem Lösungsmittel behandelt, liefert 2 Flecken, von denen zufolge Chromatogramm 1 keiner mit Adenin, Guanin, Cytosin oder Thymin identisch sein kann.

Fraktion SII gibt unter den gleichen Bedingungen 4 Flecken, von denen einer noch Cytosin sein kann, dagegen *keiner Thymin*. Man kann daraus schliessen, dass die ursprüngliche Fraktion II *kein gemischtes Dinukleotid* enthalten kann, das wir durch Spaltung mit verd. HCl erhalten wollten. Die Hydrolyse von DNS muss also offenbar mit noch milderen Bedingungen durchgeführt werden.

Die Fraktionen SIII und SIV kommen in diesem Chromatogramm nicht zum Vorschein.

DNS-Spaltung durch Ameisensäure

60 mg DNS wurden bei Raumtemperatur in 1 ml 98%iger Ameisensäure auf dem siedenden Wasserbad gelöst. Um die Hydrolyse während des Versuches zu verfolgen, wurden jede dritte Minute Versuchstropfen entnommen und auf ein Chromatogr.papier gebracht. Nach einer Hydrolysenzeit von 48 Minuten wurden alle Flecken gemeinsam chromatographiert.

Chromatogramm A

Papier: Whatman nr. 1. *n*-Butanol-Ameisensäure-Wasser, 77 : 10 : 13 [2]. Entwickler: $\text{Hg}(\text{NO}_3)_2/\text{Na}_2\text{S}$.

Substanzen: Versuchstropfen nach 3, 6, 9 ... Min.

Ergebnis: Alle Tropfen konnten entwickelt werden und befanden sich auf der Startlinie.

Beim ersten Tropfenversuch (3 Minuten) zeigten sich Andeutungen von 2 Flecken, nämlich bei $R_f = 0.21$ und $R_f = 0.35$. Beim 6-Min.-Versuch hatte die Stärke dieser Flecken zugenommen; sie waren nun deutlich sichtbar.

Bei den folgenden Versuchen (9–48 Min.) war die Stärke der Flecken unverändert, was andeutet, dass bei der Hydrolyse nach 9 Minuten keine weitere Substanzbildung erfolgt.

Identifizierung der Flecken R_f 0.21 und 0.35 des Chromatogramms A

Chromatogramm B

Papier, Flüssigkeit und Entwickler wie im Chromatogramm A.

Substanzen: Adenin, Cytosin, Guanin, Thymin, $\text{PO}_4\text{H}(\text{NH}_4)_2$ und 48-Min.-Versuch.

Ergebnis: Die beiden Flecken des Chromatogramms A sind identisch mit *Adenin* und *Guanin*.

Phosphat konnte durch den gleichen Entwickler *nicht* hervorgerufen werden.

Folgende R_f -Werte wurden erhalten: Adenin 0.35, Guanin 0.21, Cytosin 0.30, Thymin 0.58. 48-Min.-Versuch 0.00, 0.21 und 0.35.

Im 48-Min.-Versuch müssen also im Flecken 0.00 Cytosin u. Thymin in gebundener Form vorhanden sein.

Nach der 48-Min.-Hydrolyse der DNS durch Ameisensäure bei 100° wird Adenin und Guanin abgespalten; eine weitere Abspaltung diese Basen tritt

anscheinend nicht ein. Dagegen konnte eine Substanz festgestellt werden, die im Chromatogramm nicht wanderte. Diese Substanz isolierten wir in folgender Weise:

Die 48-Min.-Hydrolyse-Lösung wurde mit dem 5fachen Volumen Äthanol versetzt, wodurch eine sehr schwerlösliche schwach rotbraune Substanz von amorphem Charakter ausfiel. Die ganze Flüssigkeit wurde zenfrifugiert, der ungelöste Teil wurde in 5 ml Äthanol aufgeschlämmt und wieder zweimal zentrifugiert.

Wir erhielten nun 7 mg Substanz, die in 0.175 ml konc. Ameisensäure aufgelöst wurde. Ein Tropfen der Lösung wurde nun chromatographiert um zu ermitteln, ob Adenin und Guanin durch die Fällung mit Äthanol entfernt worden war. Verfahren wie in Chromatogramm A.

Es konnte nun festgestellt werden, dass das gesamte Adenin und Guanin entfernt worden war. Der Rest der Substanzlösung wurde nun im Bombenrohr 30 Min. auf 175° erhitzt, wodurch nach G. R. Wyatt (Biochem. J. 48, 584, 1951) sämtliche Basen in Freiheit gesetzt werden.

Nach dem Erhitzen hatte sich ein schwarzes Pech auf dem Boden des Gefäßes gebildet (zersetzte Desoxyribose und Phosphorsäure) und oberhalb desselben eine fast farblose Lösung, die chromatographisch wie oben untersucht wurde.

Chromatogramm

Folgende R_f -Werte wurden beobachtet:

		Hydrolyse-Lösung (175°)
Adenin	0,36	0,00 kaum bemerkbarer Flecken
Guanin	0,10	0,08
Cytosin	0,29	0,19
Thymin	0,60	0,29
		0,36 kaum bemerkbarer Flecken
		0,46 » » »
		0,60

Das aus der ursprünglichen Hydrolyse-Lösung (100°) durch Äthanol-Fällung erhaltene hydrolysierte Produkt enthält also Guanin, Cytosin und Thymin, dagegen nur Spuren von Adenin.

Der Hauptteil des Adenins wird zufolge Chromatogramm A bei der Hydrolyse mit Ameisensäure bei 100° schnell abgespalten. Teilweise wird das Guanin ebenfalls abgespalten, ist aber noch in gebundener Form übrig und wird durch Alkohol-fällung erhalten.

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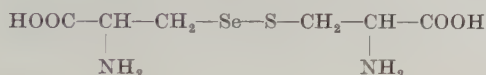
Uppsala 1961. Almqvist & Wiksells Boktryckeri AB

1,9-Diamino-3,5,7-trithianonane-1,9-dicarboxylic acid and 1,9-diamino-3,7-diselena-5-thianonane-1,9-dicarboxylic acid

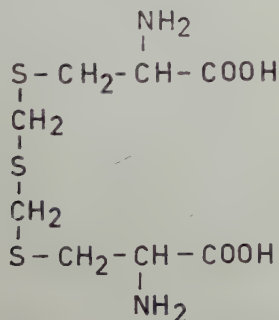
By GÖRAN ZDANSKY

With 2 figures in the text

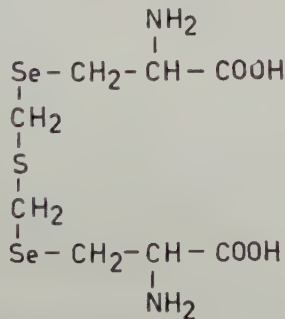
In connection with work in progress on selenium amino acids it was deemed of interest to investigate the possibility of preparing a mixed selenium-sulphur amino acid. Since it can be assumed that, e.g., sulphur-selenium cystine



would readily disproportionate according to $2 \text{R-S-Se-R} \rightleftharpoons \text{R-S-S-R} + \text{R-Se-Se-R}$, it was decided to isolate the atoms of sulphur and selenium, respectively, by an interposed methylene group. On account of their thioether and selenoether linkage, respectively, these new amino acids will exhibit close connection with lanthionine and djenkolic acid. The mixed selenium-sulphur amino acid especially ought to be of great interest in biochemistry, since to the knowledge of the present author no acid of this type has so far been prepared and examined. Recently Frankel and Gertner [1] have prepared some homologues of djenkolic acid of which one contained sulphide as well as ether linkages.



I



II

With their low solubility the new amino acids, for which the names 1,9-diamino-3,5,7-trithianonane-1,9-dicarboxylic acid (I) and 1,9-diamino-3,7-diselena-5-thianonane-1,9-dicarboxylic acid (II), respectively, are proposed, resemble greatly cystine and lanthionine. An interesting observation has been made concerning the difference in reactivity of cystine and selenocystine towards potassium cyanide. Schöberl [2] has availed himself of the instability of the cystine in presence of potassium cyanide for the isolation of lanthionine from wool treated with alkalis, and has thus in an elegant way solved the difficult problem of the separation of cystine from lanthionine. In control experiments with about 10 mg of L-cystine and the same quantity of inactive selenocystine the cystine was found to have disappeared completely after 45 minutes' treatment with potassium cyanide according to Schöberl, while the selenocystine, after acidification with dilute acetic acid was again precipitated in unaltered condition. This has been checked with IR. This process might supply a simple method for the separation of certain selenium amino acids from their sulphur analogs, when natural substances are to be treated.

Experimental

The required 1,3-dichloro-2-thiapropane has been prepared according to Bloch and Höhn [3], and distilled in vacuum. Bp. 85–86°/69 mm, $n_{20}^D = 1.5347$. The β -benzylselenocystine required for the syntheses of II was obtained in good yield by acid hydrolysis of Se-benzyl-N-acetylselenocystine which had been prepared according to [4].

1,9-Diamino-3,5,7-trithianonane-1,9-dicarboxylic acid, I

3.5 g (1/50 mole) L-cysteine hydrochloride monohydrate were suspended in 60 ml 99.5 % ethanol. 1.85 g sodium were added in small pieces during stirring. After all sodium had been dissolved 1.31 g (1/100 mole) 1,3-dichloro-2-thiapropane diluted with 10 ml 99.5 % ethanol were added in a single portion. The reaction mixture was left standing for 1½ hours during stirring. Then 50 ml water were added, and the pH adjusted to 6 by the addition of hydrochloric acid. After some minutes the copious white precipitate was sucked off, washed with water, and dried in the air. The crude product obtained in this way was suspended in 100 ml water. Concentrated ammonia was added drop by drop, until an opalescent solution was obtained which was filtered. To this solution was added 1.3 g potassium cyanide, after which it was allowed to stand for 1 hour in a closed vessel in ordinary room temperature. After this time the solution was neutralized with glacial acetic acid, whereupon the mixture was left in a closed vessel for 20 minutes in order that the precipitation of the amino acid might be complete. Then the precipitate was filtered off by suction, and washed, first twice with 15 ml water, then with twice 15 ml ethanol, and, finally, with 15 ml ether. The amino acid free from disulphide obtained in this way was left to dry in the air over night, and was then dried over phosphorus pentoxide in a vacuum exsiccator. The acid crystallizes in the form of small rosettes consisting of thin leaf-shaped crystals.

Yield 2.20 g (73 %). At 220° the substance assumes a yellow tint. With rising temperature the colour becomes gradually darker. At 270° it is pure black, yet without having fused. With regard to solubility this amino acid much resembles cystine, the

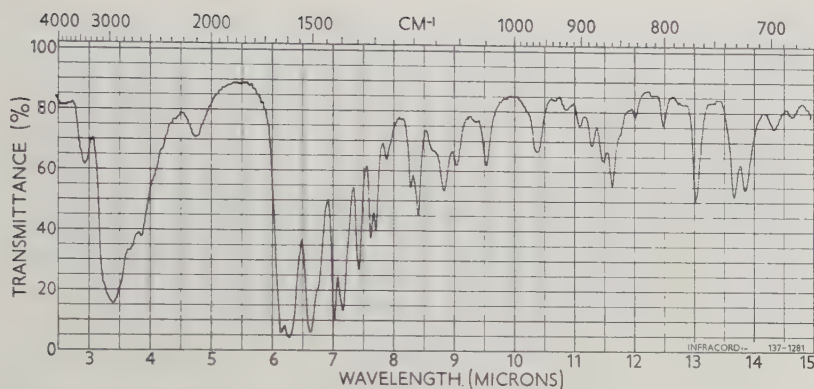


Fig. 1. IR-spectrum of 1,9-diamino-3,5,7-trithianonane-1,9-dicarboxylic acid.

feeble solubility of which in water is well known. $R_f = 0.37$ using circular filter paper technique upon Munktell No. 3 with *n*-butanol, acetic acid and water (4:1:5).

Analysis:

Found: % S: 32.00; 32.04; 31.92; 32.03. % N: 9.24; 9.25.

Calculated: % S: 32.02. % N: 9.32.

0.0751 g dissolved in 1-*N* HCl to 10.00 ml: $2\alpha_D^{25} = -0.96^\circ$. $[\alpha]_D^{25} = -64^\circ$.

IR-spectrogram see Fig. 1.

In order to test the optical purity of the L-cysteine hydrochloride a sample was oxidized with air and Fe^{3+} to L-cystine, and the specific rotation of the latter determined.

0.0997 g dissolved in 1-*N* HCl to 10.00 ml: $2\alpha_D^{25} = -4.24^\circ$. $\alpha_D^{25} = -213^\circ$.

1,9-Diamino-3,7-diselena-5-thianonane-1,9-dicarboxylic acid, II

4.1 g β -Benzylselenocysteine were suspended in about 60 ml liquid ammonia. Small pieces of sodium were added, until the blue colour lasted for 10 minutes. The reaction mixture was shielded from atmospheric humidity and carbon dioxide by means of tubes with soda lime, through which the ammonia was allowed to evaporate after completion of the debenzylation. After evaporation of the ammonia the last traces were removed by connecting the apparatus to an evacuated exsiccator containing a bowl with concentrated sulphuric acid. After a short while this evacuation was interrupted by the filling of the apparatus with nitrogen. To the dry remainder 1.10 g (calc. 1.05 g) 1,3-dichloro-2-thiapropane diluted with 20 ml 99.5 % ethanol were added. The reaction mixture was slightly warmed up by the heat of reaction, and became turbid through precipitated sodium chloride. After $2\frac{1}{2}$ hours the mixture was heated for a short time to about 50° , and the alcohol distilled off. The remainder was dissolved in 25 ml water, and concentrated hydrochloric acid was added dropwise to acid reaction. The solution was extracted twice with carbon tetrachloride. From the water-phase the amino acid was precipitated by careful addition of NaOH and sodium acetate. The amino acid was sucked off, washed with a little ice-water and then with alcohol. Reprecipitation gave a pure white product. Yield 2.4 g. Chromatography using circular filter paper technique upon Munktell No. 3 with *n*-butanol,

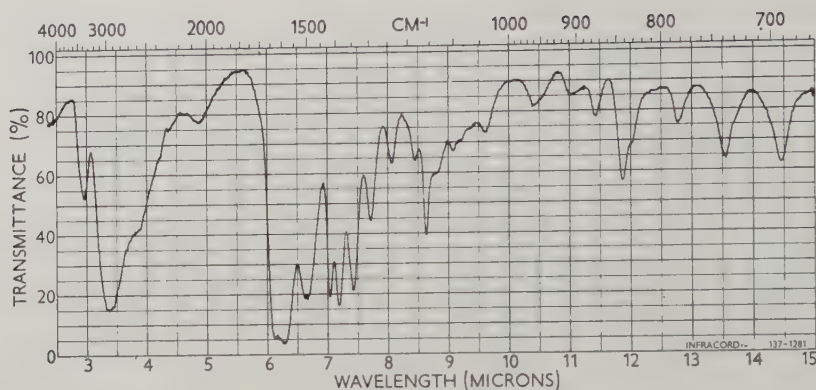


Fig. 2. IR-spectrum of 1,9-diamino-3,7-diselena-5-thianonane-1,9-dicarboxylic acid.

acetic acid and water (4:1:5) showed only one component with $R_f = 0.37$. At 168–170° the substance begins to darken, and decomposes with evolution of gas at 193–195°.

Analysis:

Found: % Se: 39.9; 39.4. % N: 6.89; 6.88 (6.97; 6.96).

Calculated: % Se: 40.1. % N: 7.11.

Note: In the presence of approximately the same amount of selenium the analysis of *p*-amino-benzoic acid gave 10.09 and 10.13 % N. The calculated value is 10.22 %. The values corrected for this decay of ammonia are given above within brackets.

IR-spectrogram see Fig. 2.

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The author wishes to express his gratitude to Professor Arne Fredga for his kind interest in this work. For the analysis I am indebted to Dr. Arthur Bengtsson and to Dr. Lilly Gustafsson of the Department of Analytical Chemistry, University of Uppsala. A grant from A.B. Astra, Södertälje, is gratefully acknowledged.

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Spectroscopic studies on enols. Part III¹

Dehydroacetic acid

By STURE FORSÉN and MARTIN NILSSON

With 5 figures in the text

SUMMARY

Infrared and proton magnetic resonance spectra of dehydroacetic acid (I) and its methyl enol ether confirm the complete enolisation of (I) and indicate that only one enol—the α -pyrone form (I *b*)—is formed. The intramolecular hydrogen bond is somewhat weaker than in the β -tricarbonyl compounds previously investigated in this series.

In the conjugated analogue (II) the strength of the hydrogen bond is markedly increased.

In previous parts of this series the hydrogen bonding in some enolised β -tricarbonyl compounds was investigated by infrared and proton magnetic resonance methods. The enol proton resonance shift and the infrared chelate carbonyl absorption in general gave concordant results in the estimation of the relative strength of the hydrogen bonds. The PMR spectra also, in some cases, gave information regarding the presence of more than one enol form [1].

The present paper deals with the investigation of dehydroacetic acid (I, 3-acetyl-6-methyl-2H-pyran-2,4(3H)-dione) using essentially the same methods. As additional unsaturation has been found to have a profound influence on the hydrogen bonding in β -triketones the analogue (II) was also investigated.

The chemistry of dehydroacetic acid has been reviewed by Henecka [2]. Previous investigations on dehydroacetic acid include studies on the ultraviolet spectra of the parent compound and its ethyl enol ether leading to the conclusion that dehydroacetic acid is completely enolised in ethanol solution [3]. Infrared [4] and Raman spectra [5] have been published without discussion.

Due to its antimicrobial properties dehydroacetic acid is widely used as a preservative but it is also known to affect the central nervous system and the metabolism of higher animals [6, 7].

Experimental

Methods

The proton magnetic spectra were recorded on a Varian V-4300 NMR spectrometer operating at 40 Mc/s and equipped with a Varian K-3519 field homogeneity

¹ Part II. Acta Chem. Scand. 14, 1333 (1960).

control unit. The measurements were made at $22 \pm 1^\circ$. The shifts were determined against an internal tetramethylsilane standard and are given as τ -values according to Tiers [8]. For conversion to the δ_{aq} values previously used in this series the following relation is approximately valid: $\delta_{\text{aq}} = \tau - 5.19$.

The infrared spectra were recorded on a Perkin Elmer No. 21 instrument with a sodium chloride prism. The measurements were taken in *ca.* 0.1 *M* solutions in chloroform using 0.1 mm cells. The solvent absorption was compensated. As most of the compounds were but sparingly soluble in carbon tetrachloride the spectra in this solvent were recorded for saturated (IR) or even supersaturated solutions (PMR).

Materials

Carbon tetrachloride was dried over phosphorus (V) oxide. The chloroform was freed from ethanol although the latter, apparently, had little influence on the results.

Commercial dehydroacetic acid (Schuchardt) was recrystallised from acetic acid and sublimed, m.p. 113–114°.

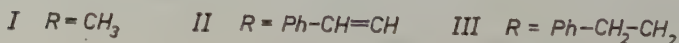
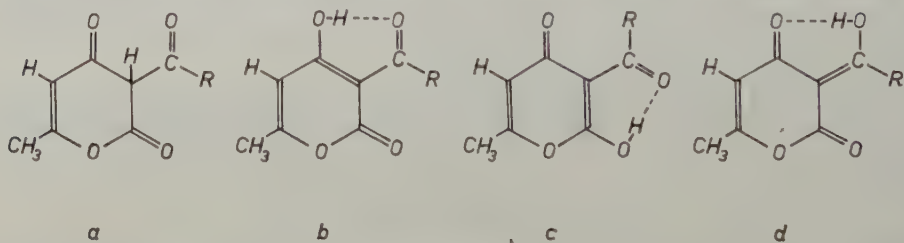
Dehydroacetic acid methyl ether was prepared by treatment of dehydroacetic acid in dry ether with a slight excess of diazomethane at room temperature. The product was recrystallised from dry ether, m.p. 93–95°. It was very sensitive to moisture and was kept in vacuum over silica gel. (Lit. m.p. 91° [9].) Ultraviolet absorption in methanol: λ_{max} 310 m μ , ϵ 9150.

3-Cinnamoyl-6-methyl-2H-pyran-2,4(3H)-dione (II) was prepared according to Wiley *et al.* [10]. It was recrystallised from methanol and sublimed, m.p. 132–135° (lit. m.p. 130–132°).

3-Hydrocinnamoyl-6-methyl-2H-pyran-2,4(3H)dione (III) was prepared in 70 % yield by hydrogenation of (II) over palladised charcoal in ethanol. Recrystallisation from cyclohexane and distillation at 0.001 mm gave colourless prisms, m.p. 78–80° (Found: C 69.3; H 5.3. Calc. for $\text{C}_{15}\text{H}_{14}\text{O}_4$: C 69.7; H 5.5.)

Results

The PMR spectrum of dehydroacetic acid in chloroform solution is shown in Fig. 1. The chemical shifts are close to those obtained in carbon tetrachloride, but due to the poor solubility in this solvent it was difficult to detect the spin-spin splitting. The spectrum is simple and signals due to the enolic proton (1), the proton in the 5-position (2), the acetyl protons (3) and the protons of the 6-methyl group (4) are



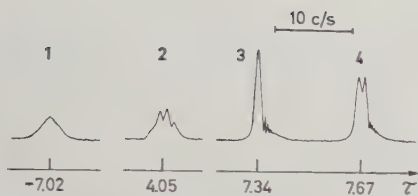


Fig. 1.

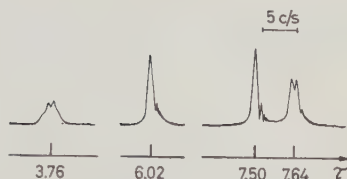


Fig. 2.

Fig. 1. PMR spectrum of dehydroacetic acid in chloroform solution (mole fraction, $x = 0.05$). The chloroform resonance is omitted. Peaks 1 and 2 are recorded with a larger gain (ca. 2 times) than the high field peaks.

Fig. 2. PMR spectrum of dehydroacetic acid methyl ether ($x = 0.05$). The peak at $\tau = 3.76$ is recorded with a gain ca. 1.5 larger than for the other peaks.

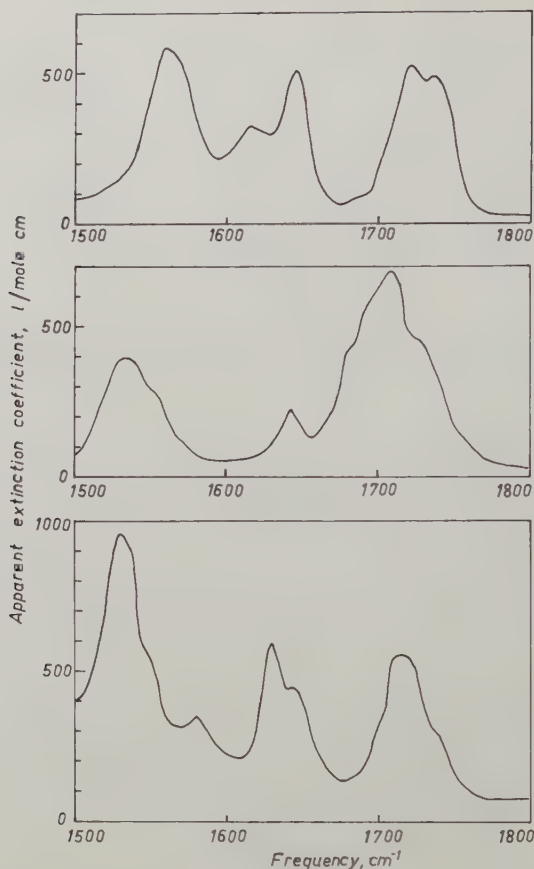
readily recognised. In carbon tetrachloride signal 1 occurred at $\tau = -7.05$. The observed spin-coupling constant for the protons of the 6-methyl group and the proton in the 5-position ($J = 0.75 \pm 0.10$ c/s) is somewhat lower than the corresponding constants in *trans*-crotonaldehyde [11], 5-methyl-5-thiolen-2-one [12] and thymoquinone [13]. The coupling confirms the assignment of signals 3 and 4. No additional splitting, which could indicate the presence of more than one enol form was observed. It is to be remembered, however, that the PMR spectrum cannot reveal the simultaneous occurrence of very easily interconvertible enols such as types *b* and *d*.

In the spectrum of the methyl enol ether (Fig. 2) the position of the signals was somewhat altered. The new signal at $\tau = 6.02$ is due to the methoxyl protons. The splitting of one methyl signal and that of the 5-proton is again observed, $J = 0.70 \pm 0.10$ c/s. The similarities of the PMR and the UV spectra of the enol and the enol ether confirm that they have the same principal structure.

Part of the infrared spectrum of dehydroacetic acid in chloroform solution is shown in Fig. 3. The spectrum conforms with those previously reported. The spectrum in carbon tetrachloride was very similar in the $1500\text{--}1700\text{ cm}^{-1}$ region, but the band slightly above 1700 cm^{-1} was not completely resolved. The spectrum of the solid (in a potassium bromide disc) closely resembled the ones for the solutions. The spectrum of the methyl ether is shown in Fig. 4. The interpretation of these spectra was aided by the recent investigations by Jones *et al.* on the infrared spectra of unsaturated lactones [14].

In Fig. 3 the double peak at ca. 1730 cm^{-1} is considered due to the lactone carbonyl of an α -pyrone form. The assignment is supported by the splitting of the peak and its sensitivity to solvents. The position is in agreement with the absorptions of α -pyrone and 4-methoxy-6-methyl-2H-pyran-2-one [14, 15]. 2-Methoxy-6-methyl-4H-pyran-4-one has no strong absorption above 1700 cm^{-1} but gives strong bands at 1677 and 1692 cm^{-1} [15]. The peaks at 1550 and 1640 cm^{-1} occur in other simple α -pyrones although they are then less intense, cf. [14]. The infrared spectra thus clearly indicate that dehydroacetic acid enol and its methyl ether are derived from the α -pyrone form.

The peak at 1615 cm^{-1} in the spectrum of dehydroacetic acid disappears on methylation. A comparison of the integrated absorption in the $1500\text{--}1800\text{ cm}^{-1}$ region shows only a minor decrease for the ether. The peak near 1700 cm^{-1} in the spectrum of the ether is considerably stronger than in dehydroacetic acid and it can therefore be concluded that the 1615 cm^{-1} peak in Fig. 3 represents the absorption of the



Figs. 3-5. Part of the infrared spectra of dehydroacetic acid (I), dehydroacetic acid methyl ether and 3-cinnamoyl-6-methyl-2H-pyran-2,4(3H)-dione (II) in chloroform solution.

chelated carbonyl group. In Fig. 4 this absorption is changed and corresponds to a normal conjugated carbonyl absorption.

The conjugated analogue (II) showed enol proton resonance at $\tau = -8.29$ in carbon tetrachloride and at $\tau = -8.20$ in chloroform solution. The *trans*-cinnamoyl pattern was similar to those described in Part I. The signals from the 6-methyl group and the 5-proton showed the same spin coupling as those above, $J = 0.60 \pm 0.10$. This confirms the proposed structure, which was somewhat uncertain in view of the known reactivity of the 6-methyl group in related pyrones, cf. [15].

The infrared spectrum, shown in Fig. 5, contains new peaks due to the conjugated benzene ring and the *trans*-ethylenic double bond. The lactone peak and the 1646 cm^{-1} peak occur almost unchanged but the intensity of the band near 1530 cm^{-1} is considerably increased. It seems probable that the chelate carbonyl absorption is included in this band but its exact position cannot presently be defined.

The spectra of the hydrogenation product (III) proved to be similar to those of dehydroacetic acid; the enol proton resonance occurred at $\tau = -6.95$ (carbon tetrachloride) and the infrared chelate carbonyl absorption at 1615 cm^{-1} .

Discussion

The spectroscopic data show that only one enol of dehydroacetic acid is present in the solid and in solutions in polar as well as in non-polar solvents. The infrared data provide strong evidence for the 4-hydroxy- α -pyrone form (Ib) and it is evident that the enol ether is derived from this form also. Form (Id) cannot be excluded but is considered less favoured. The α -pyrone form has previously been found to predominate in triacetic lactone (6-methyl-2H-pyran-2,4-dione); in solution this is present almost exclusively in the 4-hydroxy- α -pyrone form although methylation may give ethers derived from the 2-hydroxy- γ -pyrone form also [15].

The hydrogen bond in the enol of dehydroacetic acid, as measured by the infrared chelate carbonyl frequency and the PMR enol proton shift, is not as strong as in the β -tricarboxyl compounds previously investigated ($\nu_{\text{CO}} 1580 - 1515\text{ cm}^{-1}$, $-\tau = 7.4 - 8.9$). The additional unsaturation in compound (II), however, as in other cyclic β -tricarboxyl compounds, increased the strength of the hydrogen bond.

At first sight it is surprising that the hydrogen bond in dehydroacetic acid is weaker than in the diacetoacetic esters described in Part II. This is probably due to the influence of the 4-5 double bond both directly on the adjacent chelate system and indirectly *via* the lactone grouping.

Magnetic anisotropy effects may influence the enol proton resonance field and make comparisons with other data inadequate. Although the magnitude of the effects in this case are difficult to estimate it is to be noted that in *o*-hydroxyazobenzene derivatives the effect on the enol proton shift has been estimated to give an enol proton resonance at *ca.* 1 p.p.m. lower field than is actually representative of the hydrogen bond [16].

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S. FORSÉN AND M. NILSSON, *Spectroscopic studies on enols. Part III*

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On octa-(arylsilsesquioxanes), $(\text{ArSi})_8\text{O}_{12}$

I. The phenyl, 4-tolyl, and 1-naphthyl compounds

By KJELL OLSSON and CHRISTINA GRÖNWALL

With 1 figure in the text

In a previous paper by one of us [1] the oligo-(alkylsilsesquioxanes), $(\text{RSiO}_{1.5})_n$, and oligo-(arylsilsesquioxanes), $(\text{ArSiO}_{1.5})_n$, known then, were listed, and the literature concerning these compounds was given. In this list $(n\text{-C}_5\text{H}_{11}\text{SiO}_{1.5})_8$ and $(\text{C}_6\text{H}_5\text{SiO}_{1.5})_8$ were overlooked. These substances had been prepared shortly before by Sprung and Guenther [2] in a study of the hydrolysis of *n*-amyl- and phenyl-triethoxysilane. Müller, Köhne and Sliwinski [3] later obtained the octameric parent compound, $(\text{HSiO}_{1.5})_8$, in less than 1 % yield by hydrolyzing trichlorosilane in 80 % sulphuric acid in the presence of hexamethyldisiloxane. Dr. K. Larsson at the Inorganic Department of this Institute quite recently completed an X-ray study of $(\text{HSiO}_{1.5})_8$ [4], $(\text{CH}_3\text{SiO}_{1.5})_8$ [5], and most of the other known octamers [6]. The present work was briefly mentioned by Fredga [7] in a review of adamantane-like compounds. Thus oligo-(arylsilsesquioxanes) have so far been prepared in three instances, Ar being in all cases phenyl:¹

1. By Barry *et al.* [11], who treated an ethereal solution of phenyltrichlorosilane with excess of water. After removing the hydrogen chloride formed they left a solution of the hydrolysis product in benzene-ethanol at room temperature in the presence of a trace of potassium hydroxide. Since they found $n = 6.5$ (average) ebullioscopically, and since X-ray powder photographs showed the substance to be nonisomorphous with several mutually isomorphous octa-(alkylsilsesquioxanes), Barry *et al.* concluded $n = 6$.

2. By Sprung and Guenther [2], who rearranged a high polymer obtained by alkaline hydrolysis of phenyltriethoxysilane. They assumed that the rearrangement, which was carried out under various conditions, was catalyzed by traces of alkali, retained by the polymer. Ebullioscopic determinations afforded $n = 7.0$ (average), i.e. $n = 6$ or $n = 8$. A comparison of the far infrared spectrum with the spectra of several hexa- and octa-(alkylsilsesquioxanes) showed $n = 8$ to be the most probable alternative.

3. By Olsson [1], who refluxed phenyltrichlorosilane with strong methanolic

¹ According to Andrianov, Dzhenchel'skaia, and Petrashko [8] crystalline $(\text{C}_6\text{Cl}_5\text{SiO}_{1.5})_8$ was prepared through hydrolysis of pentachlorophenyltrichlorosilane by Andrianov and Odinets [9]. However, nothing of this was mentioned in the latter paper. In a following paper by Andrianov and Odinets [10] this hydrolysis was in fact described but the product was a polymer; either acid or alkaline medium was used.

hydrogen chloride containing about 2 % of water. From the X-ray powder photograph the cell dimensions were calculated, and, in combination with the density, afforded $n = 8$.

The solubility data and other physical properties reported for the three oligo-(phenylsilsesquioxanes) are so similar, that we suspected the substances to be identical. The crystals were described by Barry *et al.* as "rods" or "crystals having a square outline" and by Sprung and Guenther as "small plates with rectangular or square faces". The same crystal form is therefore indicated in both cases. Olsson, however, considered his crystals as being of rhombic shape. In the present investigation the experiments described in [1], [2], and [11] were carefully repeated. After one recrystallization from pyridine each of the three products was examined under the microscope. This showed them all to be mixtures containing crystals of both the square and rhombic types, with the former usually predominating. Further crystallization from methylene chloride of any of the three recrystallized products usually gives only crystals of the square type. On crystallization from hot quinoline, crystals of the rhombic type are the main or sole product.

X-ray powder photographs were taken of two samples, one containing only crystals of the square type, the other composed entirely of "rhombic" crystals. The two photographs were entirely different. Finally, X-ray powder photographs were taken of the three crude products obtained according to [1], [2], and [11]. The three last-mentioned photographs could be represented exactly as combinations of the two first-mentioned photographs. The crude product of Sprung and Guenther exhibited some weak, additional reflexions, which, however, disappeared after the recrystallization from pyridine. Very little substance was lost in this recrystallization. When the crude product of Barry *et al.* was recrystallized from pyridine, a large part of it was retained in the mother liquor, from which only amorphous material could be isolated.

From these facts it may be concluded that the three oligo-(phenylsilsesquioxanes) reported in [1], [2], and [11] are actually identical, although the substance appears in two polymorphic modifications. The crude product of Sprung and Guenther evidently contains traces of another crystalline substance, while that of Barry *et al.* contains amorphous impurities.

Single crystals of both polymorphic forms and of various other octasilsesquioxanes, described below or in [1], have been examined by Larsson [6]. Some of his results are reproduced in Table 1. These are the types of unit cell and the numbers, N , of formula units $\text{RSiO}_{1.5}$ or $\text{ArSiO}_{1.5}$ in the cell, calculated from the cell dimensions and densities. In those compounds which belong to the trigonal crystal system either a hexagonal unit cell containing three molecules or a rhombohedral unit cell containing one molecule may be chosen, as exemplified by the high-temperature form of the *n*-propyl compound in [6]. Rhombohedral unit cells are used throughout in this paper, since their relation to the numerous triclinic cells (which here can be regarded as pseudorhombohedral) is more easily recognized.

The data for the methyl and ethyl compounds are in accordance with those already given in [1]. On the basis of an X-ray powder photograph with only few and diffuse reflexions, a rhombohedral unit cell was assigned to the phenyl compound in this reference. This assignment proved to be erroneous, however, and the data given in [1] for the phenyl compound are therefore irrelevant. In the present instance examination of single crystals showed those of the square type to be monoclinic and those of the rhombic type to be triclinic. The new results are consistent with $n = 4$, $n = 8$, or (for the monoclinic form) $n = 16$, but not with $n = 6$. In view of the ebullioscopic

Table 1. Unit cells and cell contents of some compounds $(\text{RSi})_8\text{O}_{12}$ and $(\text{ArSi})_8\text{O}_{12}$ according to Larsson [6].

R/Ar	Unit cell	N
Methyl	Rhombohedral	8
Ethyl	Rhombohedral	8
Isopropyl	Rhombohedral	8
n-Propyl	Rhombohedral ^a	8
n-Propyl	Triclinic ^b	8
n-Butyl	Triclinic	8
Phenyl (rhombohedral type)	Triclinic	8
Phenyl (square type)	Monoclinic	16
<i>o</i> -Nitrophenyl	Triclinic	8
4-Tolyl	Orthorhombic	32
1-Naphthyl	Triclinic	16

^a Above -1° .

^b Below -1° .

data given in [2] and [11], the phenyl compound must therefore be octameric, with two molecules per unit cell in the monoclinic form and one molecule in the triclinic form.

The solubility of the phenyl compound in boiling methylene chloride was found to be 1.73 g in 100 ml by Sprung and Guenther. In view of this value and the high molecular weight, 1034, the elevation in boiling point observed by Barry *et al.* can have been no more than about 0.05° , which appears too little for a decision between the alternatives $n = 6$ and $n = 8$, in spite of their use of the precision method of Kitson, Oemler, and Mitchell, Jr. [12]. The second argument of Barry *et al.*, based on X-ray powder photographs, in favour of a hexameric structure is irrelevant, for Larsson's data in Table 1 show that several crystal systems are compatible with an octameric structure.

The methods used in [2] and [11] give the phenyl compound in very high yields but require several weeks. On the other hand, the method used in [1] is rapid but the yield is only 9 %. Several modifications of the procedures described in [1] and [11] were therefore tried, including changes in the kind and amount of catalyst, in the kind and amount of solvent, in the amount of water (cf. [1]), and in temperature. For the former procedure the yield could be raised to 25 % and for the latter (by simply refluxing the solution), to 74 %, the reaction time being 3 days in both cases.

Several modifications of both procedures were now applied to some other aryltrichlorosilanes. With neither method were crystalline products obtained from 3-chlorophenyl-, 4-bromophenyl-, and 4-anisyl-trichlorosilane. 4-Tolyl- and 1-naphthyl-trichlorosilane, however, were converted in 2 days into the corresponding octa-(arylsilsesquioxanes) in 19 and 11 % yield respectively, when Olsson's method was used. The method of Barry *et al.* afforded no 4-tolyl compound and only traces of the 1-naphthyl compound. The latter was also prepared in 52 % yield in 21 hours by a modification of the method of Sprung and Guenther. That this method and Olsson's method give the same 1-naphthyl compound is shown by identical X-ray powder photographs, infrared spectra, melting points, and other physical properties.

Both the 4-tolyl and 1-naphthyl compounds are colourless, crystalline, and somewhat more soluble in the usual solvents than the phenyl compound, though much less soluble than the ethyl and higher alkyl compounds. The 1-naphthyl compound melts at about 343° with slow decomposition; the 4-tolyl compound decomposes at about 400° without true melting. Their octameric status is proved by Larsson's X-ray data in Table 1, combined with cryoscopic determinations. A run of the 4-tolyl compound in the ultracentrifuge to sedimentation equilibrium showed this substance to be absolutely homogeneous but gave a molecular weight corresponding to $n = 6.5$. This result is hard to explain. However, it is possible that the nearly saturated toluene solution used was far from ideal.

As is evident from the work reported here and earlier, the octamer is generally the main or sole crystalline product when oligosilsesquioxanes are prepared. Although other oligomers may have escaped isolation in some cases, there seems to be little doubt as to the preferential formation of the octamers. The two tetramers described by Wiberg and Simmler [13] appear to be the only exceptions. Their structure is based on ebullioscopic data, whereas the X-ray investigation of the *t*-butyl compound by Schwab, Grabmaier and Simmler [14] does not exclude an octameric structure. In both [13] and [14] the adamantane-like tetrameric structure was erroneously regarded as the only structure possible to construct for oligosilsesquioxanes. The insolubility of the tetramers in benzene and methylene chloride, which are among the best solvents for the octamers, is hard to understand.

The reason why the preparative methods used often result in low yields or fail completely is not clear. Sprung and Guenther have interpreted the rearrangement of their high polymer to oligo-(phenylsilsesquioxanes) in a convincing way as a result of successive bimolecular nucleophilic substitutions on silicon, induced by traces of alkali. Theoretically, such substitutions might transform the polymer into an infinite number of molecular and ionic species of widely varying molecular weight. After sufficient time equilibrium should be established among the original polymer and all these molecules and ions. If any of the molecular species is sufficiently sparingly soluble, its equilibrium concentration, under the experimental conditions, may exceed its solubility and cause its precipitation. The equilibrium is then displaced in favour of the precipitated substance. This will continue to separate, until the total concentration of dissolved material is so small that the equilibrium concentration of the precipitated substance in the liquid phase equals its solubility.

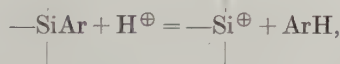
The same mechanism clearly operates in the method of Barry *et al.*, which differs significantly from that of Sprung and Guenther only in the way of preparing the intermediate polymer. Barry *et al.* have already interpreted their closely related "thermal" process in an analogous manner. The extraordinarily good yield of the phenyl compound in the method of Barry *et al.* may then be connected with its slight solubility. The methyl compound, which is still less soluble, has been prepared by Barry *et al.* in the same way. The amorphous impurities obtained when the phenyl compound is prepared according to their original method are practically absent when the experiment is carried out at reflux temperature and the yield of the pure octamer is improved. The higher temperature seems necessary, therefore, in order that the solubility of no other substance than the very slightly soluble octamer shall be exceeded.

As mentioned above, the 1-naphthyl compound was obtained in much higher yield by the method of Sprung and Guenther than by that of Barry *et al.* In both methods the rearrangement of the intermediate polymer was carried out in acetone

solution in exactly the same way. Furthermore, when the methanol used as solvent in the preparation of the polymer in the method of Sprung and Guenther was exchanged for acetone (in order to simplify the procedure), no 1-naphthyl compound at all was obtained. These facts show that the nature of the intermediate polymer is critical. In order to explain this we must assume that the equilibrium mentioned above is attained within the reaction time only among molecules and ions with mutually related structures. Thus a polymer with a ladder-like structure should rearrange easily to octameric or hexameric molecules, as described by Sprung and Guenther, whereas a polymer prepared in a different way (or with a different type of aryl group) may have a structure which is either more apt to produce other oligomers or is unsuitable for production of any oligomer at all.

In Olsson's method a mechanism based on a similar equilibrium among an initially formed polymer and its structurally related rearrangement products is again possible since hydrogen chloride is known to rearrange siloxanes under certain conditions. However, the rather low yields hardly support such a view.

When the phenyl compound is prepared by Olsson's method, the yield is much improved if the concentration of hydrogen chloride is lowered. Accordingly, one cause of reduced yield in Olsson's method might be cleavage of the Ar-Si bonds by the hydrogen chloride. This well-known so-called protodesilylation reaction has been studied systematically by Benkeser *et al.* [15]. They consider it to be an electrophilic, aromatic substitution,



in which the reaction rate depends on Ar in the way expected from this assumption. The yields in Olsson's method might be interpreted on this basis. The unsuccessful attempts with the two halophenyltrichlorosilanes can hardly be explained in this way, however, since at least the 3-chlorophenyl group would be split off from silicon less readily than a phenyl group.

It should also be pointed out that the octa-(arylsilsesquioxanes) and their precursors are evidently much more resistant towards hydrogen chloride than are the aryltrialkylsilanes investigated in [15]. The three strongly electronegative oxygen atoms bound to each silicon atom render the Ar-Si bonds considerably less polar, and the inherently electropositive silicon atoms will be less prone to acquire a positive charge. The relation between the stabilities of phenyltrichlorosilane and diphenyl-dichlorosilane towards electrophilic agents as found by Yakubovich and Motsarev [16] supports this argument.

In some experiments to be published later various octa-(arylsilsesquioxanes) were refluxed with a large excess of bromine overnight. In no case was any noticeable Ar-Si cleavage observed, whereas bromine entered the aromatic nuclei to a varying degree. Thus the 2-thienyl compound, which will be described in a later paper, afforded the perbromo derivative in nearly quantitative yield. Since even the reactive thiophene nucleus remains attached to silicon under these severe conditions and in spite of the strong evolution of hydrogen bromide, Ar-Si cleavage can hardly be important in Olsson's method.

The great resistance of the octa-(arylsilsesquioxanes) towards Ar-Si cleavage by electrophilic agents was also demonstrated by the nitration of the phenyl compound

in fuming nitric acid without perceptible Ar-Si cleavage. Nitration of other monoaryl-silicon compounds usually requires special techniques, cf. e.g. Benkeser *et al.* [17, 18], to avoid extensive Ar-Si cleavage. As far as we know, however, compounds in which the silicon atom carries three strongly electronegative atoms or groups have not been examined before, because most of them will be converted into polymeric mixtures in the nitration medium.

As was briefly mentioned in [1], analysis and molecular weight establish the formula $(\text{NO}_2\text{C}_6\text{H}_4\text{Si})_8\text{O}_{12}$ for the nitration product. The molecular weight is based on Larsson's X-ray data in Table 1. Owing to very diffuse X-ray reflexions these data are less accurate [6] but nevertheless show the substance to be octameric or tetrameric. The tetrameric alternative is excluded by the result obtained cryoscopically. Since the nitro derivative was obtained in fairly high yield from the phenyl compound, already shown to be octameric, an oligomer other than the octamer would, of course, be very improbable.

Since a nitro group strongly deactivates the benzene ring against further substitution, it is self-evident that each phenyl group carries one and only one nitro group. Attempts to determine the orientation of this nitro group in relation to the silicon atom (by heating the nitro compound with bromine-water in a sealed tube) were unsuccessful. Of course, this orientation may vary not only among different molecules but also among different benzene rings within the same molecule. A more or less random orientation is indicated by the diffuse X-ray reflexions and the relatively poor crystallization tendency. In the formula given in [7] the *para* orientation has been chosen tentatively. In a similar way the 4-tolyl compound was converted into a crystalline nitro derivative, for which the analyses indicate the formula $[(\text{NO}_2)_2\text{C}_7\text{H}_5\text{Si}]_8\text{O}_{12}$. In [7] the positions 3 and 5 have been tentatively assigned to the nitro groups. Since this dinitro-4-tolyl compound has not been obtained quite pure as yet, it will be described more closely in a later paper. Nitration of the 1-naphthyl compound afforded no crystalline product.

Some attempts to reduce the nitrophenyl compound to the amino derivative met with no success. The technique used by Benkeser *et al.* [17, 19] was also tried. Attempts to oxidize the methyl groups of the 4-tolyl compound to carboxyl groups also failed.

Experimental

Materials

The aryltrichlorosilanes (all of which have been reported earlier) were prepared from the corresponding arylbromides by the Grignard method. The Grignard reagent was added with stirring to a 50 % excess of silicon tetrachloride, and the mixture left at room temperature overnight without stirring. The magnesium halides were filtered off with suction and washed with petroleum ether (b.p. 40–60°). The solvents and the excess of silicon tetrachloride were removed from the combined filtrate and washings at atmospheric pressure, and the residue was distilled to dryness *in vacuo* without column. The distillate was finally fractionated *in vacuo* through a short column. However, 1-naphthyltrichlorosilane was prepared according to the modification of Sanin [20]. The boiling points of the fractions used and the yields are collected in Table 2.

4-Bromophenyltrichlorosilane was also prepared by bromination of phenyltrichlo-

Table 2. Boiling points and yields of ArSiCl_3 .

Ar	B. p.	Yield in %
Phenyl	75–77°/11 mm	53
3-Chlorophenyl	106°/13 mm	33
4-Bromophenyl	125–126°/17 mm	30
4-Anisyl	122–124°/10 mm	56
4-Tolyl	95–97°/10 mm	65
1-Naphthyl	150–154°/10 mm ^a	60

^a M. p. 55–57° after recrystallization from petroleum ether (b. p. 40–60°; 3 ml/g; cooling to –20°).

rosilane according to Yakubovich and Motsarev [21]. The product had the same boiling point as that obtained by the Grignard method. The two samples were used separately with the same results.¹

1-Bromo-3-chlorobenzene was prepared from 3-chloroaniline according to Hartwell [22], b.p. 192–194°. The remaining arylbromides and the 3-chloroaniline were commercial samples of good grade; that of 4-bromotoluene was "free from isomers".

1-Naphthyltrimethoxysilane was prepared from 1-naphtyltrichlorosilane according to [20], b.p. 162–164°/10 mm, m.p. 35½–37° after recrystallization from methanol (3 ml/g; cooling to –20°). Evidently, the melting point, 28°, reported in [20] is too low.

*General procedure for preparation of octa-(arylsilsesquioxanes)
according to Olsson's method*

To 475 ml of 95 % ethanol, 0.100 mol of aryltrichlorosilane is added with stirring for 5 min. The clear solution is refluxed with stirring for 48 h. The mixture is filtered hot and with suction. The crude product is washed once with methanol, dried, recrystallized, washed with acetone, and dried again. The yield is measured at this point. (Usually more or less of the crude product adheres to the walls of the flask and may be rinsed out conveniently with the solvent used in the recrystallization.) For analysis a sample was recrystallized once more, powdered, and dried *in vacuo* at 100° for 1 h.

Octa-phenylsilsesquioxane), (C₆H₅Si)₈O₁₂

Method of Barry et al. (cf. [11]). To a solution of 21.2 g (16.1 ml, 0.100 mol) of phenyltrichlorosilane in 200 ml of ether, 25 ml of water is added at such a rate, with stirring and cooling in ice-water, that the temperature remains below 10°. The ether layer is separated, washed thoroughly several times with water, then once with aqueous sodium bicarbonate and finally once with water, and dried for a short time over sodium sulphate. The ether is distilled off on a water bath maintained at 70°. The viscous residue is dissolved in 750 ml of benzene. After addition of 150 ml of absolute ethanol and 1.0 ml of 1.01 N ethanolic potassium hydroxide, the clear solution is refluxed with stirring for 72 h. The mixture is filtered hot and with suction. The

¹ The preparation and investigation of the sample obtained by the Grignard method were carried out by Miss Britta Björkén.

Table 3. Corresponding values of x , Z , x^2 and $^{10}\log (Z/x)$ at sedimentation equilibrium in the ultracentrifugation of octa-(4-tolylsilsesquioxane).

x in cm	Z in μ	x^2 in cm ²	$^{10}\log (Z/x)$
6.550	107	42.90	-2.787
6.600	117	43.56	-2.751
6.650	127	44.22	-2.719
6.700	139	44.89	-2.683
6.750	152	45.56	-2.647
6.800	166	46.24	-2.612
6.850	180	46.92	-2.580
6.900	196	47.61	-2.547
6.950	214	48.30	-2.512
7.000	235	49.00	-2.474
7.050	257	49.70	-2.438
7.100	282	50.41	-2.401
7.150	308	51.12	-2.366
7.200	337	51.84	-2.330
7.250	370	52.56	-2.292

precipitate is washed with ether and air-dried. Weight 11.6 g. It is recrystallized from 1500–1750 ml of pyridine, washed with ether, and dried. Yield 9.6 g (74 %).

Olsson's method (cf. [1]). The general procedure is followed, but the solution is refluxed for 72 h. Recrystallization is effected from 200 ml of chlorobenzene (or 475 ml of pyridine). Yield 3.2 g (25 %).

Large crystals of the monoclinic form are obtained easily when a solution of octa-(phenylsilsesquioxane) in methylene chloride is allowed to evaporate slowly. Fairly large crystals of the triclinic form are obtained when a quinoline solution saturated near the boiling point is cooled slowly.

Octa-(4-tolylsilsesquioxane), (4-CH₃C₆H₄Si)₈O₁₂

The general procedure of Olsson's method is followed. For recrystallization the crude product is dissolved in 40 ml of boiling methylene chloride. The solution is mixed thoroughly and rapidly with 80 ml of acetone and left in the refrigerator overnight. The octamer separates as beautiful rods, some of which are more than 1 cm long. Yield 2.7 g (19 %).

C₅₆H₅₆O₁₂Si₈ (1146) calc. C 58.70, H 4.93, M 1146;
found C 58.71, H 4.86, M 1030.

The molecular weight was determined cryoscopically in naphthalene and corresponds to $n = 7.2$ formula units 4-CH₃C₆H₄SiO_{1.5} in the molecule. The substance was also run in a Svedberg oil-turbine ultracentrifuge. Details concerning the construction and operation of this instrument have been given by Boestad, Pedersen, and Svedberg [23]. The course of the sedimentation was followed by the Lamm scale method [24]. The substance was examined in 1 % toluene solution at 22.0° and at a speed of 700 r.p.s. and run to sedimentation equilibrium (about 20 h). The molecular weight, M , was obtained from a formula first used by Lamm [25]:

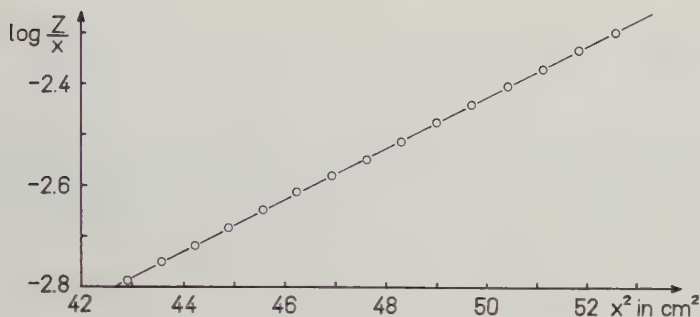


Fig. 1.

$$M = \frac{2RT}{(1 - V_0)\omega^2 \log e} \cdot \frac{d \log (Z/x)}{d(x^2)}$$

For the meaning of the symbols see [23]. Fig. 1 shows a plot of $\log (Z/x)$ against x^2 . The numerical data are collected in Table 3. The strict linearity proves that the solute is monodisperse. The value $d \log (Z/x)/d(x^2) = 0.0512 \text{ cm}^{-2}$ was obtained from the slope of the line. The partial specific volume, V , was determined by the pycnometer method, cf. Kraemer [26], in toluene solutions of four different concentrations and at $22.0 \pm 0.01^\circ$. V was found to be independent of the concentration, and $1 - V_0 = 0.321 \pm 0.005$. The formula above then gives $M = 930$ corresponding to $n = 6.5$. The accuracy of this result should be 5–10 % [27].

Octa-(4-tolylsilsesquioxane) is practically insoluble in water, alcohol, and acetone but usually soluble to some extent in less polar solvents except ligroin and petroleum ether. One ml of boiling methylene chloride dissolves 70–80 mg. Crystals formed in the presence of acetone or ligroin rapidly become opaque on drying. Evidently, these solvents are loosely incorporated into the crystal lattice. Other solvents, e.g. ethyl acetate and toluene, do not behave in this way, and give rods which remain transparent on drying. The decomposition point, about 400° , was measured with a chromel–alumel thermocouple.

Octa-(1-naphthylsilsesquioxane), $(1\text{-C}_{10}\text{H}_7\text{Si})_8\text{O}_{12}$

*Method of Sprung and Guenther.*¹ To a solution of 24.8 g (0.100 mol) of 1-naphthyltrimethoxysilane in 475 ml of methanol are added 16 ml of triethylamine and 34 ml of water.² The clear solution is heated to boiling, when it becomes turbid immediately. It is then refluxed for 3 h (1–2 h may be sufficient). The supernatant liquid is decanted hot and discarded. The sticky residue is washed once with methanol and dissolved in 160 ml of acetone. In a few minutes some octamer, evidently formed directly in the hydrolysis, begins to separate. After addition of 2.7 ml of triethylamine and 5.3 ml of water, the mixture is refluxed with stirring for 18 h and then filtered hot with suction. The microcrystalline precipitate, consisting of almost pure octamer, is washed with acetone. It weighs 9.9 g dry. It is dissolved in 75 ml of boiling pyridine. After cooling to about 60° the solution is mixed thoroughly and rapidly with 225 ml

¹ Part of this procedure was worked out by Miss Britta Björkén.

² Practically the same yield is obtained with 95 ml of methanol, 3.2 ml of triethylamine and 9.0 ml of water.

of acetone to which 35 ml of water has been added, and left in the refrigerator overnight. The crystals are collected, washed with acetone, and dried. Yield 9.3–9.4 g (52 %).

If the 1-naphthyltrimethoxysilane is refluxed directly with acetone, triethylamine, and water, no precipitate will form. If the intermediate polymer is prepared through hydrolysis of 1-naphthyltrichlorosilane in ethereal solution by the method applied to phenyltrichlorosilane by Barry *et al.*, only a very small precipitate will form during the subsequent refluxing with acetone, triethylamine, and water. However, the isolation of 1-naphthyltrimethoxysilane may be avoided in the following way, although the yield will be lower:

To a solution of 58 ml of triethylamine in 485 ml of methanol, 26.2 g (0.100 mol) of 1-naphthyltrichlorosilane is added with stirring for 5 min. From the addition of 34 ml of water onwards the original procedure is followed. Yield 6.6–6.7 g (37 %).

Olsson's method. The general procedure is followed, but the 95 % ethanol is exchanged for (100 %) 1-propanol; and after the addition of the 1-naphthyltrichlorosilane (conveniently as supercooled liquid), 65 ml of water is run in rapidly. Recrystallization is effected from 16 ml of pyridine, 48 ml of acetone, and 7 ml of water as described above in the method of Sprung and Guenther. Yield 2.0 g (11 %).

$C_{80}H_{56}O_{12}Si_8$ (1434) calc. C 67.00, H 3.94, M 1434;
found C 66.99, 66.55, H 4.09, 3.91, M 1270.

The molecular weight was determined cryoscopically in naphthalene and corresponds to $n = 7.1$ formula units $1-C_{10}H_7SiO_{1.5}$ in the molecule. As to solubility and occurrence of crystal solvent, the substance resembles the 4-tolyl compound, but it is less soluble in methylene chloride. The approximate amount dissolved by 1 ml of boiling solvent is for pyridine 135 mg, for chlorobenzene 65 mg and for carbon disulphide 50 mg. Fairly large crystals of rhombic shape (destroyed on drying) will form slowly if the water is omitted in the recrystallization described above. When placed in a melting point apparatus preheated to 337° and adjusted to a rate of heating of 3° per min, the substance forms a clear melt at 343°. If this determination is repeated as quickly as possible, the same sample will melt at about 335° owing to partial decomposition. (The temperatures are corrected.)

Octa-(x-nitrophenylsilsesquioxane), (x-NO₂C₆H₄Si)₈O₁₂

To 6.0 ml of fuming nitric acid is added with stirring and cooling in ice-water 1.3 g (0.010 mol/8) of octa-(phenylsilsesquioxane) in small portions. Each portion dissolves completely in a few seconds with a fizzing sound. After all has been added, the clear solution is poured on to 50 g of ice. When the ice has melted, the very faintly yellow precipitate is collected, washed with water and then with methanol, and air-dried. Weight 1.6 g. It is recrystallized from 100 ml of glacial acetic acid. A rather small amount of insoluble material is removed by rapid filtration of the hot solution. The product is washed with methanol and dried. Yield 0.9 g (50 %). For analysis the product was recrystallized once more from glacial acetic acid and dried *in vacuo* at 100° for 1 h.

$C_{48}H_{32}N_8O_{28}Si_8$ (1394) calc. C 41.37, H 2.31, N 8.04, M 1394;
found C 41.35, H 2.39, N 7.87, M 1370.

The molecular weight was determined cryoscopically in phenol and corresponds to $n = 7.9$ formula units $x\text{-NO}_2\text{C}_6\text{H}_4\text{SiO}_{1.5}$ in the molecule. The substance is usually obtained as an almost colourless microcrystalline powder. Small crystals of rhombic shape will form slowly if petroleum ether is added carefully to an acetone solution until a slight opalescence persists after mixing. Large crystals have not been obtained. The nitro compound is practically insoluble in water, alcohol, ether, carbon tetrachloride, carbon disulphide, petroleum ether, and ligroin; slightly soluble in acetone and boiling glacial acetic acid; moderately soluble in benzene, chloroform, and pyridine; and easily soluble in dioxane, ethyl acetate, methylene chloride, and boiling 2-butanone. It melts at about 325° (dec.).

X-ray powder photographs

The photographs were taken in a focusing camera of the Guinier type with Cu $K\alpha$ radiation.

SUMMARY

The three oligo-(phenylsilsesquioxanes) reported in the literature [1, 2, 11] are shown to be identical and octameric, although polymorphic. The substance is obtained in 74 % yield in 3 days. The crystalline 4-tolyl and 1-naphthyl analogues have been synthesized; but attempts to prepare the corresponding 3-chlorophenyl, 4-bromophenyl, and 4-anisyl compounds failed. The preparative methods are discussed. A crystalline octanitro derivative of the phenyl compound is described.

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Laboratory of Organic Chemistry, Chemical Institute, University of Uppsala, October 1960.

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Some reactions of succinylated collagen

By K. H. GUSTAVSON

Introduction

For elucidation of the mechanism of reactions of collagen with various agents, particularly those which are irreversibly fixed by the protein, investigations of the behavior of modified collagens have been fruitful [1]. Thus, specific groups, for instance the ϵ -amino groups of the residues of the lysines, have been replaced by other groups (diazotization), or inactivated by substitution (acetylation). Further, the carboxyl groups have been inactivated by esterification (methylation). As a rule, these inactivation and replacement reactions do not change the charge distribution of the protein appreciably, although its isoelectric point is generally displaced, for instance, by diazotization [2], acetylation [3], and methylation [4].

It has been claimed that by the condensation of succinic acid anhydride (SAA) with the ϵ -amino groups of the lysine residues of globular proteins [5], the positively charged ammonium group is replaced by a group carrying a free, negatively charged carboxyl group. This would be expected to induce a high density of negative charge on the succinylated protein molecule. Moreover, the electrostatic repulsion induced would result in unfolding of the structure, which should be expected to be manifested in swelling of insoluble proteins, such as collagen.

Succinylated globular proteins have been used in studies of antigenic problems, particularly albumins and globulins, as well as gelatin and modified gelatins [5-7]. Since this mode of altering the lysine-amino groups apparently is specific for the ϵ -amino groups and, moreover, since it promises to hold interesting possibilities as to protein reactivity, the succinylation of collagen and the reactivity of the resulting product have been included in the author's series of investigating variously modified skin proteins [1]. In view of the key-position of the cationic chromium complexes among the aniono-philic metal complexes of the transition group of elements, a protein substrate with a part of its cationic groups replaced by anionic groups, in this instance the carboxyl ions, which complex with electropositive chromium, should appear to offer interesting possibilities. Earlier, unpublished attempts to use α -bromo-acetic acid [8] for introducing the carboxymethyl group on the amino group of collagen were not entirely satisfactory due to the inadequate specificity of this reagent. Glyoxylic acid has been employed for replacing the amino group with the carboxyl group in collagen, making use of the aldehydic function of this aldehydic acid. This carboxymethylation was apparently less effective [9].

Materials and Methods

Standard Hide Powder (Lyon) and the thin middle layer of the *corium* of calfskin (split), served as collagen substrates. The skin had received a light alkaline pretreatment (liming). The skin was subsequently restored to the isoelectric state (pH 6.0). The isoelectric states were attained at pH 5.5 and 6.0 for the hide powder and intact skin, respectively.

For the succinylation of the hide powder, the method of Habeeb *at al.* [5] has proven satisfactory after minor modifications. Portions of 30 g of air-dry hide powder (= 26.4 g of collagen) were soaked in 300 ml of 2.8 per cent solution of sodium bicarbonate overnight. With continuous, vigorous stirring of the swelled hide powder sludge, a quantity of 12.0 g of finely powdered succinic acid anhydride was added gradually for four hours. The system was held in the pH range 8–8.5 by gradual addition of one-molar solution of sodium hydroxide. The stirring was continued for a further four hours. The batch was then left overnight and the mixing continued for a further two hours. The surplus solution was removed by suction filtering. In the early series, the greatly swelled, slightly alkaline SAA-hide powder was brought to neutral reaction by means of acetate buffers (pH 7.0–7.5), followed by washing for removal of the sodium acetate. In the following main series, the succinylated hide protein was equilibrated in citrate buffer solutions at pH 2.8, the isoelectric point of the modified collagen found during the early orientation.

The SAA-hide powder, equilibrated at pH 7.5 and washed, was in a highly swelled state. It contained 1.5 per cent ash, on the dry weight basis, present in the form of sodium held by the carboxyl groups. By bringing it into the isoelectric state (pH 2.8), the swelling was largely eliminated and the alkali salts removed (de-ashed). The treated hide powder was finally dehydrated in acetone and vacuum-dried at 40°C. It was then left in the air for equilibration. For the control (Blank), portions of hide powder weighing 30 g were treated as described, except that no SAA was added. For the succinylation of the skin collagen, specimens of strips, measuring 5 mm × 30 mm of ca. 1 mm thickness, weighing 100 g (= 30 g of collagen) were equilibrated at pH 8.5 in 100 ml of sodium bicarbonate solution. The succinylation was performed as described for the hide powder. However, serious difficulty was experienced in obtaining a uniform product, particularly on processing calfskin. The chief reason being the marked swelling of the more compact skin pieces, whereby the even penetration of the agent applied was obstructed, resulting in the interior layers of the skin being insufficiently succinylated. However, the thin split (*corium*) of sheepskin (degreased), possessing great porosity, could be succinylated uniformly and exhaustively. The main part of this investigation was carried out with the succinylated hide powder specimens, restored to the isoelectric state.

For the evaluation of the degree of succinylation, the total nitrogen content of the succinylated collagen was determined by the Kjeldahl method. Further, since its acid-binding capacity is a measure of the degree of inactivation of the acid-binding (basic) protein groups, the acid binding power of the original and the modified specimens of hide powder was determined by the following method [10]. Portions of the air-dry hide powder, corresponding to 500 mg of collagen, were equilibrated with 10.0 ml of 0.1 *N* solution of hydrochloric acid, containing 4.0 w/v per cent of sodium chloride, functioning as a swelling depressant (4 hours at 20°C with intermittent shaking). The quantity of acid removed by the protein from the solution, with final pH of 1.3–1.4, was obtained by difference from the data of the acid content

Table 1. Characteristic data on the collagen specimens.

(SAA-hide powder in isoelectric state.)

No.	Type	% N	% Collagen	% fixed SAA	Acid- binding capacity	Mequiv. basic groups inactivated	pH of isoelectric point
1.	Original hide powder	18.01	100.0	0.0	0.92	0.00	5.5-5.6
2.	SAA-hide powder	17.36	96.3	3.7	0.56	0.36	2.7-2.8
3.	Sheepskin pelt (<i>corium</i>)	16.92	100	0.0	0.86	0.00	6.3-6.5
4.	SAA-sheepskin pelt (<i>corium</i>)	16.37	96.8	3.2	0.54	0.32	2.9-3.0

Note: The shrinkage temperature, T_s , of the sheepskin collagen was lowered from 56°C to 48°C, or by 8°C, on its succinylation.

of the original and the residual solutions, correcting for the moisture content of the original specimens and their degree of hydration, i.e., by collagen tightly bound water which does not function as solvent for the hydrochloric acid (about 20 per cent of the weight of the protein [11]).

Further, the pH values corresponding to the isoelectric point of each specimen, the original and modified collagens, were determined by means of the dyestuff technique [12] applying anionic (acid) dyes: Methanil yellow, Crystal Ponceau 6R and Orange II, all sulfo-acid compounds; and cationic (basic) dyestuffs, Methyl violet 6B, and Methylene blue, in 0.1 per cent aqueous solution of adjusted pH (buffers). The hide powder specimens (50 mg) were equilibrated with the buffer solutions either in acetic acid-sodium acetate, or citric acid-sodium hydroxide, or hydrochloric acid) in the pH range 2.0-7.0. The specimens were then treated with solutions of the dyestuffs of pH values and type of buffer corresponding to those used for the pH adjustment of the various collagen samples. For the corium of the sheepskin (16.9 per cent total N, on organic substance; 12.0 per cent hydroxyproline, figured on the same basis), in the original and succinylated states, the pH values corresponding to their isoelectric points were obtained by measuring the dialysis potential by Staverman's method [13]. Maurer and Lebovitz [6] report the isoelectric range of pH 2.5-3.0 for the succinylated gelatin.

Table 1 contains the pertinent data for the characterization of the original collagen and the succinylated specimens, brought to the isoelectric state (pH 2.8). The figures are based on ash-free dry weight, i.e., organic substance. These preparation contained less than 0.1 per cent of ash, on dry substance basis. The acid binding capacity is expressed in mequiv. HCl fixed by one g of collagen.

Agents used for interaction with collagen

The effect of the exchange of an electro-positive group for an electro-negative one in the collagen on its general reactivity, particularly on the irreversible binding of different types of chromium complexes, was the main theme of this investigation. It was expected that for agents specifically bound to the hide protein by its carboxylic group, the succinylated collagen should yield information about the state of the carboxylic group introduced into the protein by the succinylation. The cationic

chromium complexes, such as the sulfato-chromium compounds in the form of an equilibrated solution of the 67 per cent acid chromium sulfate, its composition corresponding to the empirical formula: $\text{Cr}_2(\text{OH})_2(\text{SO}_4)_2 \cdot \text{NaSO}_4$ at a concentration of 1.0 equiv. per liter Cr, possess such a specificity. This type of basic chromium sulfate has been described in a previous paper [14]. In chemical reactions for modifying collagen, the occurrence of secondary alterations, apart from those of the direct combination of the agent with other protein groups, has also to be considered and ascertained. Thus, for instance, by changes in the milieu, e.g., by applying extreme pH values, swelling of the protein may be incurred. This may in its turn lead to rupture, or weakening of valency bonds of the type of hydrogen bonds, particularly on adjacent keto-imide groups, generally considered to be the main stabilizing cross-linkages of the collagen structure.

The irreversible binding of polyphenols of the type represented by vegetable tannins (mimosa bark extract) by collagen is to the keto-imide groups mainly, by multipoint formation of hydrogen bonds; with the phenolic hydrogen functioning as the donor and the keto oxygen of the $-\text{CO} \cdot \text{NH}-$ link of the protein as the acceptor of such a bond [15]. By rupture of such hydrogen bridges, acceptor sites are made available for binding of the tannins. Hence, the degree of fixation of the vegetable tannins may serve for an evaluation of any changes in the reactivity of the keto-imide group of the protein.

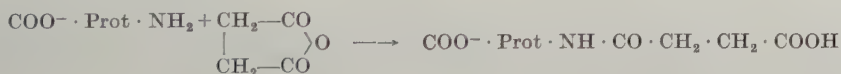
Further, solutions of extremely basic chlorides and perchlorates of chromium, such as the 33 per cent acid compounds, with composition corresponding to the empirical formula: $\text{Cr}_2(\text{OH})_4\text{X}_2 \cdot 2 \text{NaX}$ ($\text{X} = \text{Cl}$, or ClO_4), were employed [16]. They contained appreciable amounts of aggregated chromium complexes, mainly in the uncharged state, in addition to simple cationic chromium complexes. The reaction of the cationic chromium compounds with collagen is governed by the state of the ionic protein groups, while for the main constituent, the polynuclear complexes, the degree of availability of nonionic groups in the collagen, chiefly the keto-imide groups, is determining, as shown in a previous paper [16]. Finally, a solution of sulfito-chromium sulfate, its composition corresponding to the empirical formula: $\text{Cr}_2(\text{OH})_2(\text{SO}_4)_2 \cdot 3 \text{Na}_2\text{SO}_3$ [17, 18] was included, in order to ascertain the occurrence of any alterations of the keto-imide and hydroxy groups, the latter ones particularly [19]. This solution contained the main part of its chromium in the form of uncharged complexes, 70 per cent; the rest being negatively charged [19].

Interaction. Since the technique employed has been described in detail in an earlier paper [16], it suffices to state that portions of specimens of intact collagen and succinylated collagen in an amount equal to 500 mg of protein, in fully hydrated state, were shaken for periods from 4 hours up to 28 days, at a temperature of 20°C , with 20.0 ml portions of solutions of the chromium salts, containing 0.8 equiv. Cr per liter. The vegetable tannins contained 5.0 w/v per cent of tannin (pH 5.0). The time of treatment was 120 hours. The original pH values of the solutions of the chromium salts were in the range 2.9–3.0 for the 67 per cent acid sulfate; pH 3.7–3.8 for the 33 per cent acid chromium compounds; and pH 5.5 for the sulfito-chromium sulfate. After completed interaction, the treated specimens were freed from adhering solution by washing. On the dry chromed specimens, the contents of ash, chromium and protein were determined by the conventional methods. The factor for conversion of per cent nitrogen into per cent hide substance was 5.56, and for the succinylated specimens which had been vegetable tanned: 5.76. For the chromed collagen the degree of fixation is given in mequiv. Cr fixed per g of collagen. Since the amount of

irreversibly fixed vegetable tannins is obtained indirectly as the difference between 100, and the sum of ash and protein, it is obvious that for the vegetable tanned succinylated specimens the succinyl-residue has to be included, i.e., the factor of 5.76 has to be employed. Thus, $N \times 5.76$ represents the SAA-collagen compound (nitrogen content: 17.36 per cent, on the weight of organic substance).

Results and Discussion

In the only detailed study of the reaction of SAA with proteins previously published, that of Habeeb, Cassidy and Singer [5] on globular proteins in aqueous solution, the optimal pH value for the reaction was established to be about 8, with 95 per cent of the total number of the ϵ -amino groups of bovine serum albumin reacting with SAA. At lower pH values, for instance at pH 5, no reaction occurred. With collagen, the pH range 8.0–9.0 was found to yield maximum interaction. The data in Table 1 show that the hide powder collagen had fixed 3.8 per cent SAA, on protein basis. The degree of inactivation of the basic groups, estimated from data on the acid-binding capacity of collagen in the original and the succinylated states, corresponds to an amount of 3.6 per cent bound SAA. Since the total amount of ϵ -amino groups of the lysine and hydroxylysine residues is 0.40 mequiv. g of collagen [20], the limited or maximum fixation by the ϵ -amino groups should be 4.0 per cent of SAA. Hence, about 95 per cent of the ϵ -amino groups had entered into reaction with the SAA. Maurer and Lebovitz [6] report 94 per cent inactivation of the SAA-gelatin. It should be noted that attempts of reacting the SAA in organic solvents (ethanol; acetone) with acetone-dehydrated collagen proved to be a complete failure; only a minute fixation being obtained. This failure is not unexpected, since NH_2 -groups and not NH_3^+ -groups are the sites for the reaction, as proved by the marked pH dependency of the fixation of SAA by proteins, exhibiting a sudden, very drastic increase of the binding at pH values > 8 . Under these conditions, the reaction is represented by the equation:



This equation represent the SAA-collagen in the isoelectric state. However, at pH 7.5 some ionization of the introduced carboxyl groups has to be taken into consideration.

The pH value corresponding to the isoelectric point of the native collagen, schematically written as $\text{COO}^- \cdot \text{Prot} \cdot \text{NH}_3^+$, was 5.5. The collagen which had been modified by succinylation attained the isoelectric state at pH 2.7–2.8, which implies a displacement of the isoelectric point toward the acid side of the pH scale of 2.7 units as a result of the coupling of the free ϵ -amino groups with SAA. This displacement is more than twice that effected by diazotization of collagen [2]. Hence, the introduced carboxyl group apparently exerts a very marked effect.

An interesting point, emphasized by Habeeb *et al.* [5], is that by the binding of the SAA on the protein a NH_3^+ -group will be replaced with a group with $-\text{NH} \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COO}^-$ -function at *neutral* pH. However, at the isoelectric point of the succinylated collagen, i.e., at pH 2.8, the introduced carboxyl groups are evidently present in the unionized state, further proved by the titration curves to be published in a forthcoming paper. In the case of SAA-collagen of the pH 7.5 milieu, some of the

Table 2. Effect of succinylation on the fixation of vegetable tannins by hide powder collagens.

No.	Type of tannins	Final pH	Intact collagen	% tannin fixed irreversibly by:	
				SAA-collagen of pH 7.5	pH 2.8
1.	Mimosa	4.9	47	59	45
2.	Sulfited quebracho	5.5	52	63	51
3.	Tannic acid	3.5	81	83	67
<i>Fixation of vegetable tannins by skin collagens</i>					
4.	Sulfited quebracho	5.5	43	50	43

introduced carboxylic groups are ionized. The swelling of the succinylated collagen, probably induced by the electrostatic repulsion of the introduced negatively charged carboxyl groups, should be expected to increase with augmented pH.

On the other hand, the hydrothermal stability of the SAA-collagen should be expected to decrease. This interesting problem is to be further investigated. Moreover, the reactivity of agents which have specific affinity for the carboxyl ions of the protein should also be favoured by increasing pH values of the system as the present data also prove. For agents such as the vegetable tannins, some order of pH dependency should also be expected, since by a gradually acquired negative charge of the introduced carboxylic groups, their mutual repulsion would tend to augmented swelling, and concurrently result in dislocation and rupture of hydrogen bridges. Thus, additional free coordination sites are made available to the reactant. The solutions of the succinylated globular proteins studied by Habeeb *et al.*, which were not adjusted to the isoelectric point, showed greatly increased intrinsic viscosities in spite of sharply reduced sedimentation constants, pointing to a molecular unfolding of the protein chains as the main factor. Probably also in this instance, the repellation of the negatively charged carboxyls introduced is involved. The same explanation likely applies to the lowering of the shrinkage temperature of the skin collagen by the succinylation, amounting to 8°C.

For didactic reasons the results from the series with the vegetable tannins, given in Table 2, will first be considered.

As already mentioned, the vegetable tannins rely on the oxygen of the uncompensated keto-groups of the $-\text{CO}\cdot\text{NH}$ -links of the protein for their irreversible combination with the collagen. This is decidedly so for the condensed typed of tannins, such as those of the mimosa and quebracho extracts [21]. The most striking evidence is the irreversible uptake of these tannins by certain polyamides to a degree comparable with that of collagen. In these synthetic substrates, the $-\text{CO}\cdot\text{NH}$ -link is practically the sole binding site [22, 23]. The data for series No. 1 and No. 2 show practically the same extent of binding of the tannins by the intact hide powder and the succinylated specimens of collagen in the isoelectric state.

It is conceived to be a proof of the thesis that the hydrogen bridges on the keto-imide groups of collagen are not appreciably altered by the succinylation, if the modified collagen is immediately brought to its isoelectric state. Thereby the swelling is markedly reduced. However, the binding of tannic acid, which belongs to the

Table 3. Fixation of chromium compounds by hide powder and isoelectric SAA-hide powder (pH 2.8).

No.	Type and composition of Cr-salt	Charge of Cr-complexes	Time of tanning (hrs)	Final pH	Mequiv. Cr fixed by:	
					Intact collagen	Isoelectric SAA-collagen
1.	67 % acid Cr-sulfate, $\text{Cr}_2(\text{OH})_2(\text{SO}_4)_2 \cdot \text{Na}_2\text{SO}_4$	{ Cationic (98 %)	4	2.9	3.3	2.1
2.			24	2.9	4.1	3.2
3.			96	3.0	4.8	4.3
4.			720	3.0	5.0	5.1
5.	33 % acid Cr-chloride, $\text{Cr}_2(\text{OH})_4\text{Cl}_2 \cdot 2\text{NaCl}$	{ 40 % cationic 60 % uncharged	4	3.7	3.8	2.4
6.			24	3.7	4.5	3.1
7.			96	3.8	4.7	3.7
8.			720	3.8	5.0	4.6
9.	33 % acid Cr-perchlorate, $\text{Cr}_2(\text{OH})_4(\text{ClO}_4)_2 \cdot 2\text{NaClO}_4$	{ 35 % cationic 65 % uncharged	24	3.7	4.7	3.6
10.			96	3.7	5.0	4.3
11.			720	3.8	5.2	5.2
12.	Sulfito-Cr-sulfate, $\text{Cr}_2(\text{OH})_2(\text{SO}_4)_2 \cdot 3\text{Na}_2\text{SO}_3$	{ 70 % uncharged 30 % anionic	24	5.3	5.1	7.9
13.			96	5.3	6.0	9.2
14.			720	5.4	10.4	16.4

hydrolysable class of tannins, by the isoelectric SAA-collagen is markedly impaired by the inactivation of the ϵ -amino groups. On the other hand, the SAA-collagen of the pH 7.5 milieu possesses the same affinity for tannic acid as the intact collagen (Blank). Since for this type of tannins, the basic protein groups are instrumental in binding a considerable part of the tannins, the decreased fixation of this polyphenol by the isoelectric SAA-collagen is in line with our present concept of these complicated reactions [24]. On the other hand, by the high degree of swelling of the SAA-collagen of pH 7.5 new reaction sites on the $-\text{CO} \cdot \text{NH}$ -links are made available to the tannins. This positive effect outweighs the negative effect due to the blocking of the ϵ -amino groups.

The results of the reaction of the isoelectric SAA-collagen with the chromium compounds are exemplified in Table 3. The corresponding data for the blanks

Table 4. Fixation of chromium compounds by hide powder and SAA-powder of pH 7.5.

No.	Mequiv. Cr fixed by 1 g of collagen as	
	Intact collagen	SAA-collagen
3.	4.6	4.8
7.	4.8	9.4
10.	4.9	8.1
13.	6.1	9.2
15. 67 % acid Cr-chloride, ^a [25] $\text{Cr}_2(\text{OH})_2\text{Cl}_4 \cdot 2\text{NaCl}$	3.0	3.2
16. Tetraoxalato-diol-chromiate, ^b [26]	2.9	3.3

^a 100 % cationic chromium complexes.

^b Mainly anionic complexes, and some uncharged chromium.

(buffer-treated collagen) are included. Further, Table 4 contains some typical data obtained for SAA-collagen of pH 7.5 with the same solutions on 96 hours' tanning. The amounts of chromium irreversibly fixed by the collagen are expressed in mequiv. of Cr per g of collagen. All the solutions had an initial concentration of 0.8 equiv. Cr per liter. The designation of the chromium salts in Table 4 by number refers to the numbers in Table 3.

In order to clear the ground, it is to be noted that the reaction of cationic chromium complexes, such as those recorded in Nos. 1-4 (Table 3), with collagen is markedly impaired in the initial stage by the inactivation of the basic protein groups, for instance by deamination [27], or acetylation [28], although the carboxyls specifically interact with, and bind these complexes, the basic protein groups not being directly involved. The explanation of the lowered chrome uptake is probably the following. This reaction involves the protolyzed sulfuric acid and the sulfate anions of the chromium sulfate also. By inactivation of the basic groups of collagen, the removal of sulfate ions by the protein is appreciably diminished. Since the system represents an equilibrium of the various ions, the impaired electro-neutralization of the sulfate anions by collagen retroacts on the discharge of the electropositive chromium complexes, which is lowered. Obviously an absolute condition is, that the electro-neutrality of the system is upheld. On prolonged interaction, secondary reactions of another type set in. In the isoelectric SAA-collagen, the introduced carboxyl groups are *not* ionized and hence they cannot participate in binding of cationic chromium. In the SAA-collagen of pH 7.5, on the other hand, some of these carboxyls are ionized and able to function in the chrome fixation. This compensation of the lowered chromium uptake, due to the indirect effect of the disturbed ionic equilibrium by the impaired anion-binding by the inactivated basic groups is very marked on brief periods of reaction, while on extending the time of reaction greater chrome uptake is obtained by the SAA-collagen of pH 7.5, as shown by No. 3 and No. 15 in Table 4.

As to the extremely basic chlorides and perchlorates of chromium, exemplified by Nos. 5-11 in Table 3, the minor constituent of simple, cationic chromium complexes, which are similar to those in the solutions of series Nos. 1-4, follows the same reaction mechanism as those. However, the main part of the chromium is present in the form of aggregated polynuclear complexes with very low or no electric charge [16, 29]. These complexes react with, and are fixed by the non-ionic protein groups, primarily the $-\text{CO}\cdot\text{NH}$ -links. It has been conclusively established that by the formation of additional free sites on the keto-imide groups by breaking hydrogen bridges on hydrothermal denaturation, and by treatment with hydrogen-bond breaking agents, the fixation of this type of chromium compounds is greatly increased (50-100 per cent) [30]. The chrome uptake by the isoelectric SAA-collagen (Table 3) from the solutions of these extremely basic chromium salts shows the same trend as that of the simple cationic chromium complexes. The isoelectric, succinylated collagen is not permanently swelled. Consequently the coordination capacity is not altered, and hence neither the non-cationic chrome fixation. On the other hand, the SAA-collagen of pH 7.5 which maintains a great deal of the large swelling of the succinylated collagen from the slightly alkaline milieu possesses very much greater affinity for the extremely basic chromium salts than the intact collagen does. Normally increases in fixed chromium of the order of 75-100 per cent are recorded.

The fixation of the sulfite-chromium sulfate, which is composed of non-ionic chromium complexes mainly, with a minor percentages of anionic chromium, is increased by some 50-70 per cent for both specimens of SAA-collagens, independent

of their electro-chemical state. Since this reaction involves the hydroxy groups of collagen in addition to the keto-imide groups, complicating factors probably enter. At our present knowledge, any theorization on the cause of this unique behavior is futile. However, the fact of the relatively high pH value of this system (pH 5.5) may be a factor.

The behavior of collagen with its NH_3^+ -groups turned into the $-\text{NH}\cdot\text{CO}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{COOH}$, or the $-\text{NH}\cdot\text{CO}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{COO}^-$ groups offers interesting problems. All according to the pH milieu of the final processing, i.e., whether pH 2.8, the isoelectric point of the modified collagen, or pH 7–7.5, far on the alkaline side of this cardinal point for the SAA-collagen, the reactivity of the ionic groups, the carboxyls, and above all that of the non-ionic groups of the collagen, principally the keto-imide groups, will be determined.

It has been demonstrated that succinylation of proteins such as collagen without following conditioning of the modified collagen at its true isoelectric point, keeping it at neutral reaction instead, connotes the formation of some additional carboxyl groups partly ionized and available for binding of cationic chromium complexes. Further, by preserving the swelling, making it permanent, and also sterically preventing the restoration of intermolecular hydrogen bridges, severed by the swelling induced by the succinylation, the reactivity of the nonionic protein groups, particularly the $-\text{CO}\cdot\text{NH}$ -links, is greatly enhanced. The present findings form additional evidence for the concept of the binding of cationic chromium complexes by the charged carboxyl groups of collagen, whereas aggregated polynuclear, non-cationic chromium complexes rely upon the non-ionic protein groups as the binding sites.

SUMMARY

The ϵ -amino groups of collagen, in the form of bovine hide powder, have been transformed into carboxylated groups by condensation with succinic acid anhydride in aqueous solution of pH > 8. About 95 per cent of the ϵ -amino groups were inactivated. The reactions of the modified collagen, used in the isoelectric state (pH 2.8), and in the state of neutral reaction (pH 7.5), with various chromium salts indicate, that the succinylated collagen in its isoelectric state holds the additional carboxyls in non-ionized state, and that its hydrogen bonding capacity is unaltered. The specimen brought to pH of 7.5 after the alkaline processing in the modifying reaction possesses some of the incorporated carboxyl groups in the ionized state, and moreover, still more important, the nonionic reactivity (hydrogen bonding) of the succinylated collagen is greatly augmented. The fixation of extremely basic chromium salts, such as perchlorates, and agents attached to collagen by hydrogen bonding, such as polyphenols (vegetable tannins), is appreciably increased.

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A native cobalamin-polypeptide complex from liver: investigations on the corrinoid part¹

By ANDERS HEDBOM

Introduction

As part of a research program on bound forms of vitamin B₁₂, a homogeneous cobalamin-polypeptide complex with microbiological vitamin B₁₂ activity has been isolated from ox liver. The isolation procedure as well as the general properties of the complex and the amino acid composition of the peptide part have been described earlier [1–3].

Numerous assay experiments seemed to indicate that the vitamin B₁₂ activity (anti-pernicious anaemia (APA) activity or microbiological activity) of animal liver could be explained entirely by the presence of cyanocobalamin (α -5,6-dimethylbenzimidazolylcobamide cyanide), hydroxocobalamin (α -5,6-dimethylbenzimidazolylcobamide hydroxide), or protein bound forms of the vitamin that could easily be converted to cyanocobalamin prior to the assay. For this reason, the author has not hesitated in the past to describe the complex as a cobalamin-polypeptide.

The situation changed when Weissbach, Toohey and Barker [4] identified the so-called cobamide coenzyme as present in rabbit liver. This substance which acts as cofactor for the enzymatic conversion of glutamate to β -methylaspartate had somewhat earlier been isolated from bacterial extracts. The cobamide coenzyme contains two adenylic groups and is, thus, different from the earlier reported corrinoids.

The present study was undertaken in order to compare the "cobalamin-peptide" complex with the substances found by Barker *et al.* [5]. The corrinoid part of the peptide complex was isolated in crystalline form and examined by chemical and physical methods. All results are in agreement with the conception that the peptide complex is of a true cobalamin nature, i.e. it contains α -5,6-dimethylbenzimidazolylcobamide. From the data obtained it is evident that Barker's enzyme is a different material.

Materials

The corrinoid used as starting material for the investigation was a split product obtained from the isolated "cobalamin-peptide" by treatment with cyanide and trichloroacetic acid as previously described [3]. In most cases the material consisted

¹ In this paper the I.U.P.A.C. 1959 Rules for Nomenclature in the Vitamin B₁₂ Field are followed. "Corrinoid" is thus used as a generic name for any compound containing the fundamental ring-system of vitamin B₁₂.

of pooled samples from several preparations collected during structure studies on the peptide part.

The cyanocobalamin used as reference substance was a crystalline vitamin B₁₂ preparation, lot ND0076, from Merck, Sharp & Dohme, Nederland N.V., Haarlem.

Cerium (III) nitrate, extra pure, and alumina activated and standardized according to Brockman were obtained from E. Merck, Darmstadt, Germany.

Nitroso-R-salt (1-nitroso-2-naphthol-3,6-disulfonic acid disodium salt), chloramine T, and 3-methyl-1-phenyl-5-pyrazolone were obtained from Eastman Kodak, Rochester, N.Y., U.S.A.

Corrin factors A, B and pseudovitamin B₁₂ were gifts from Dr. S. K. Kon, N.I.R.D., Shinfield, near Reading, England.

Other reagents and solvents used were commercial products of analytical grade purity.

Methods

Cobalt analyses

After drying to constant weight *in vacuo* over phosphorus pentoxide at 60°C the corrin samples were ignited in a platinum crucible in the presence of perchloric acid. The residues were dissolved in water and diluted to a known volume. For the analytical work, metal-free, redistilled water was used throughout. The cobalt content was determined with nitroso-R-salt by a colorimetric procedure according to Shipmen, Foti and Simon [6]. The absorption was determined at 530 m μ . At the wavelength of maximal absorption, 418 m μ , erroneous results may be obtained and, Beer's law was found not applicable in the lower concentration range. This is due to the fact that part of the absorption was from excess nitroso-R-salt which also absorbs at that wavelength. A cobalt standard was prepared by dissolving a known amount of CoSO₄ in water. Water-free CoSO₄ was obtained by drying CoSO₄ · 7H₂O at 500°C.

Phosphorus analyses

Determination of total phosphate was performed on samples ignited as described above, or in fact, on portions of the solution prepared for cobalt determinations. The method of Fiske and Subbarow [7] was used, with a standard solution of KH₂PO₄ as reference. The absorption at 660 m μ was measured when the colour had developed for exactly 15 min.

Cyanide determinations

A modification of Boxer and Rickards' [8, 9] method was used for the quantitative cyanide determinations. This method is based on the following reaction sequence [10]: formation of cyanogen chloride by the action of chloramine-T on cyanide; reaction of cyanogen chloride with pyridine, forming cyanopyridinium chloride, followed by hydrolytic opening of the ring with formation of glutaconic aldehyde; condensation of glutaconic aldehyde with 3-methyl-1-phenyl-5-pyrazolone to form a blue dye which is stabilized by a trace of bispyrazolone in the reagent.

Stock solutions and reagents were prepared as recommended by Boxer and Rickards [6, 7]. The bis(1-phenyl-3-methyl-5-pyrazolonè) was prepared according to the method of Knorr [11], using an excess of phenylhydrazine.

The cyano group was liberated as hydrogen cyanide from microgram quantities (2–20 m μ) of the corrinoid samples by illumination with visible light, essentially as

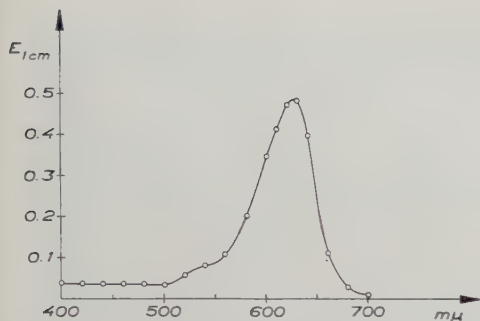


Fig. 1.

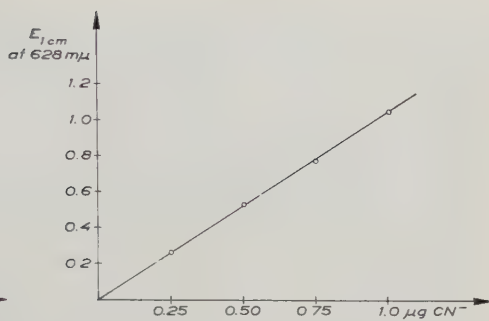


Fig. 2.

Fig. 1. Absorption spectrum of the blue colour produced by CN^- in the reaction with the pyridine-pyrazolone reagent.

Fig. 2. Calibration curve measured spectrophotometrically at a max. of $628 \text{ m}\mu$, produced by pyridine-pyrazolone reagent on different amounts of cyanide in a reaction mixture of 4.2 ml.

described by Boxer and Rickards [9]. The hydrogen cyanide was concentrated by aeration into 1 ml of 0.1 *M* sodium hydroxide solution. The flow of nitrogen gas was adjusted to 600 ml per min and the illumination and aeration continued for 2 hours at 22°C . The collection tube with the sodium hydroxide solution was cooled to 0°C , and 0.4 ml of chloramine-T phosphate reagent [8] was added dropwise. After about 2 min 3 ml of the pyrazolone-pyridium reagent [8] was added and thoroughly mixed with the solution. The tube was left at room temperature for 30 min for full colour development.

The colour intensity was measured at $628 \text{ m}\mu$, the wavelength of maximal absorption. Boxer and Rickards used $620 \text{ m}\mu$ [8, 9]. The wavelength curve is given in Fig. 1.

A standard solution of cyanide was made by dissolving potassium cyanide in water [10]. The calibration curve for a reaction mixture of 4.2 ml is given in Fig. 2. Reference samples of cyanocobalamin were always analyzed together with the unknown samples.

Ribose determinations

The orcinol method as described by Brown [12] was used for the ribose determinations. The absorption was determined at $670 \text{ m}\mu$.

Determination of refractive index

Examination by polarized light microscopy revealed that the crystals were biaxial. The order of magnitude of the refractive indices was first estimated by the degree of relief in Shillaber's immersion oil ($N_D^{25^\circ} = 1.515$). The α , β , and γ indices were then determined by means of the Becke line method in the same type of immersion liquids with appropriate indices of refraction.

Spectrophotometric measurements

Absorption spectra in ultraviolet and visible range were obtained with a Zeiss RPQ 20A recording spectrophotometer. Other spectrophotometric determinations

were performed by use of a Beckman model DU spectrophotometer. Silica cells of 1 cm light path and 4 ml capacity were generally used. Infrared-absorption spectra were determined in a Perkin-Elmer model 21 spectrophotometer [2].

Elementary analyses

Determinations of carbon, hydrogen and nitrogen content were performed by the Alfred Bernhardt Mikroanalytisches Laboratorium in the Max-Planck-Institut für Kohlenforschung, Mülheim, Germany.

Experiments and results

Purification of the starting material

The corrinoid was usually contaminated with various amounts of cyanide, phenol, ammonium, sulfate, and peptide material. To remove these impurities, the starting material was passed through a column packed with Sephadex G 25 [13] or if necessary chromatographed in a cellulose column (1.5 × 40 cm) with *n*-butanol-water as solvent system. The coloured zone was dissolved in aqueous methanol, filtered through a short column of activated alumina, and evaporated *in vacuo* to a concentrated solution. On standing or after addition of 1–2 vol acetone, the corrin derivative separated as red crystals.

Homogeneity of the preparation

As the starting material was a split product from an isolated peptide complex which itself was uniform according to the usual purity criteria for proteins [2], we had reason to believe that the preparation contained a single uniform corrinoid component. Additional evidence for purity was obtained by paper chromatography and paper electrophoresis experiments.

In ascending chromatography on Schleicher & Schüll No. 2043 paper with two different solvent systems the corrinoid appeared as a single spot detected by visible colour or by quenching of ultraviolet light.

Table 1 shows the R_F values obtained for the corrinoid compared with the values of some analogues. Solvent system I: sec-butanol saturated with water-acetic acid-10 % HCN in water (100:1:0.1); system II: the same as I but with 1.0 g sodium-tetraphenyl-borate added per litre solvent. System I is widely used for separation

Table 1. Chromatography of the corrinoid.

Solvent system I: sec. butanol saturated with water-acetic acid-10 % HCN in water (100:1:0.1). Solvent system II: The same as I with 1.0 g sodium-tetraphenyl-borate added per litre solvent.

Substance	R_F value	
	Solvent system I	Solvent system II
Corrinoid sample	0.31	0.34
Cyanocobalamin	0.31	0.35
Factor A	0.22	0.12
Factor B	0.36	0.43
Pseudovitamin B ₁₂	0.21	0.09

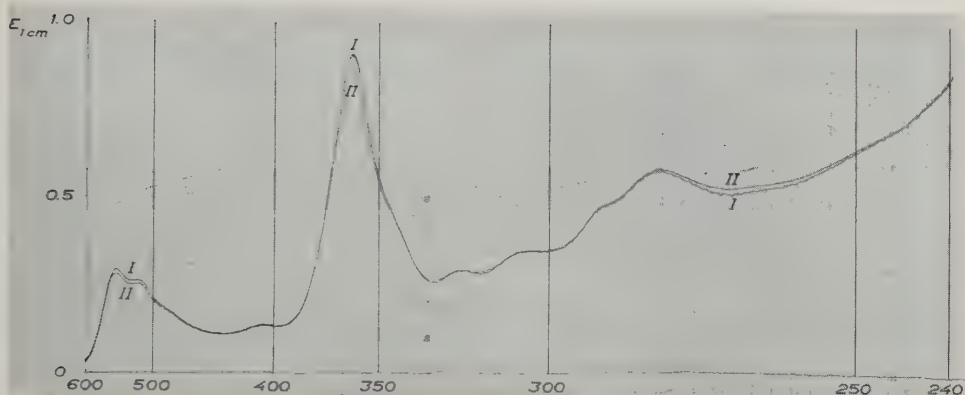


Fig. 3. Comparison of the spectra of the isolated corrinoid (I) and vitamin B₁₂ (II).

of B₁₂ analogues. System II was suggested by Friedrich, Gross and Bernhauer [14] after a thorough investigation on the influence of chromatotropic salts as the most selective solvent system for B₁₂ analogues (corrin factors).

The corrinoid behaved as a neutral substance in an electric field when applied to a Munkell 20 paper saturated with buffer solution. A field strength of 22 volts per cm was used. After correction for electro-osmosis, the rate of movement was zero in three buffer systems (I: 0.5 *M* acetic acid pH 2.6; II: 0.02 *M* phosphate, pH 6.5; III: 0.1 *M* carbonate pH 11. Only a single band or spot could be detected in each case.

In both the chromatographic and the electrophoretic experiments the conditions were such that a 4 % contamination by any of the known B₁₂ analogues would have been detected. In some doubtful cases the paper strips were subsequently analyzed by bioautography with *E. coli* [15]. No contamination or less than 0.01 % could be demonstrated, however.

Properties of the crystalline corrin derivative

The corrinoid crystallized from aqueous solution in needle-like, sometimes spherulitic, shape with clear red crystals showing pleochroism from bright red to uncoloured. On exposure to the air, they slowly lost their clear facets and turned opaque. When dried for 24 hours at 60°C *in vacuo* they lost 9 % solvent, but kept their shape fairly well. When heated in a capillary tube they darkened at 230–250°C and at a higher temperature they gradually decomposed.

Refractive index

The refractive index values have been used for the characterization of vitamin B₁₂ and crystalline B₁₂ derivatives (16). There are, however, only small differences in the values obtained for various derivatives. With small crystals it was found difficult to make reproducible accurate determinations. For this reason the method was of limited value in this case. The values obtained for the corrinoid sample as compared with those for an authentic cyanocobalamin crystal are given in Table 2.

Table 2. Refractive indices of the corrinoid as compared with cyanocobalamin.

Substance	α	β	γ
Corrinoid sample	1.619	1.647	1.659
Cyanocobalamin	1.617	1.650	1.660

Spectrum of the corrinoid

The ultraviolet and visible spectrum of an aqueous solution of the corrinoid and of a cyanocobalamin standard are given in Fig. 3. The infrared spectra of the corrinoid and the cyanocobalamin reference showed absorption bands at the same wavelength but of slightly different intensity. Both spectra indicate the identity of the corrinoid and vitamin B₁₂.

Composition of the corrinoid

Elementary analysis gave the following result: C 56.5, H 6.6, N 14.9 %. Calculated for cyanocobalamin (C₆₃H₈₈O₁₄N₁₄P Co) C 55.82, H 6.64, N 14.47 %. Results of the cobalt, phosphorus, ribose, and cyanide determinations are given in Table 3.

Table 3. Components of the corrinoid sample.

The concentration of the corrinoid was calculated from the absorption at 361 m μ . The molar extinction value of 2.80×10^4 was used.

Component	Samples analyzed	Range %	Average %	Average moles/mole
Cobalt	3	4.31-4.45	4.39	1.01
Phosphate	4	2.30-2.35	2.32	1.01
Pentose	4	8.30-8.40	8.32	0.97
Cyanide	10	1.85-1.90	1.88	0.98

Degradation studies

From the electrophoretic behavior it is evident that the corrinoid is uncharged and thus probably a "complete" factor, i.e. contains a complete nucleotide. The chemical analyses so far reported are not sufficient to reveal the nature of the nucleotide part. Spectroscopic evidence, however, indicates the identity of the corrinoid and cyanocobalamin. Drastic acid hydrolysis of the corrinoid yielded products which on paper chromatographic examination showed the same R_F values as the corresponding spots from an authentic vitamin B₁₂ sample treated in the same way. A definitive proof of the composition and structure was obtained by mild degradation of the corrinoid and quantitative analyses of the products. This was performed by hydrolysis with cerium (III) hydroxide essentially according to Friedrich and Bernhauer [17]. With this method a mild degradation of complete corrin factors to factor B, phosphoric acid, and nucleoside is possible.

The degradation reaction was performed as follows. In a 50 ml round-bottomed

flask fitted with a mechanical stirrer and a reflux condenser, 75 mg (5×10^{-5} moles) of corrinoid was dissolved in 10 ml of water. To the solution was added in turn 4 ml (200×10^{-5} moles) of a 0.5 *M* solution of $\text{Ce}(\text{NO}_3)_3 \cdot 6 \text{H}_2\text{O}$, 5 ml (500×10^{-5} equiv.) of a 1.0 *M* NaOH solution, and 0.5 ml (150×10^{-5} moles) of a 10 % HCN solution. The mixture was heated with stirring for 45 min. on a boiling water bath. After cooling of the product the pH was brought from 5.5 up to 8.5 by the addition of ammonia, and the solid material, mainly cerous hydroxide, was removed by centrifuging. The sediment was washed with a portion of aqueous ammonia which was subsequently added to the supernatant. By further addition of ammonia the pH was adjusted to 10.0 and the solution was slowly allowed to pass through a column (1.5×50 cm) packed with Dowex-2 (100–200 mesh) washed with 0.15 *M* ammonia. The red pigment was eluted with 0.25 *m* ammonia and the nucleoside first with water and finally with 0.01 *m* HCl. The fractions showing ultraviolet absorption around 280 or 360 *mμ* were concentrated to dryness *in vacuo*.

The red pigment moved as a single band in paper electrophoretic experiments at pH values of 2.6, 6.5, and 11. The mobility corresponded to that of factor B (dicyanocobinamide). Also the visible and ultraviolet spectrum coincided with that of factor B. The hydrolytic liberation of the nucleoside part with cerous hydroxide was so mild that only one coloured degradation product from the corrin nucleus could be detected.

The solid material with ultraviolet absorption at 280 *mμ* had after drying a weight of 10 mg and a melting point of 180–190°. Attempts to crystallize the material from hot water or ethanol were unsuccessful. Spot tests for carbohydrates according to Feigl [18] gave strongly positive reactions. The absorption spectrum in water, made acid to pH 2–3 by the addition of hydrochloric acid, had maxima at 278 *mμ* ($E_{1\text{ cm}}^{1\%} = 200$) and at 285 *mμ* ($E_{1\text{ cm}}^{1\%} = 203$). After addition of sodium hydroxide to a pH of 9–10 the spectrum showed maxima at 250 *mμ* ($E_{1\text{ cm}}^{1\%} = 190$), 283 *mμ* ($E_{1\text{ cm}}^{1\%} = 138$), and 290 *mμ* ($E_{1\text{ cm}}^{1\%} = 140$).

These absorption values are in fairly good agreement with those of 1- α -D-ribofuranosyl-5,6-dimethylbenzimidazole as given by Brink and Folkers [19].

To verify the identity of the nucleoside of the corrinoid with 5,6-dimethylbenzimidazole riboside, a crystalline picrate derivative was made. To an aqueous solution containing 5 mg of the substance, 0.5 ml of a concentrated aqueous solution of picric acid was added. A crystalline precipitate was immediately formed. The precipitate was isolated by centrifugation, washed twice with water, and dried. It melted at 210–213°C. Its ultraviolet spectrum showed maxima at 276, 285, and 359 *mμ*. An authentic sample of 1- α -D-ribofuranosyl-5,6-dimethylbenzimidazole picrate, obtained by degradation of cyanocobalamin in the same way, showed the same maxima and melted at 212–214°C.

From the reported data it is evident that the corrinoid part of the complex after cyanide splitting has the same properties and the same composition as the cyanocobalamin reference. The small differences found are well within the limits of experimental error. It must therefore be concluded that the corrinoid part of the peptide complex consists of α -5,6-dimethylbenzimidazolyl-cobamide; i.e. it is justified to regard the complex as a cobalamin-peptide.

Discussion

The liver depot of vitamin B₁₂ has been the object of extensive investigations. During more than twenty years from the discovery of the APA-activity in liver in

1926 to the identification of vitamin B₁₂ in fermentation broths in 1948, animal liver was the only important source of the active material. Extracts were prepared from livers of ox, horse, sheep, pig, dog, rat, mouse, guinea pig, chick, fish, and man.

The active material was found to occur as a more or less stable complex with some proteinaceous material. It was usually liberated by heating an aqueous suspension of the complex after addition of a small amount of cyanide. In some cases, however, treatment with proteolytic enzymes was necessary to secure maximal yield.

The active material obtained from ox liver by such cyanide treatment consists essentially of cyanocobalamin.

The peptide complex isolated by the present author contains no cyanide [2]. The reaction following an addition of cyanide indicates a direct linkage between the peptide and the cobalt atom.

Ford, Holdsworth and Porter [20] identified in cow liver a trace amount of factor A (2-methyladenylcobamide cyanide), and Wijmenga [21] isolated a factor WR which was subsequently shown to be a mixture of pseudovitamin B₁₂, factor A, and vitamin B₁₂.

Neglecting these very small quantities it was generally agreed that the liver depot and probably also the active form of vitamin B₁₂ was a cobalamin conjugate. Whether or not the cyanide group was usually present in the native complexes was questionable.

In 1959, Weissbach, Toohey and Barker [4] reported the presence of the so-called cobamide coenzyme in rabbit liver. This type of substance had somewhat earlier been isolated from *Clostridium tetanomorphum* and characterized as a derivative of pseudovitamin B₁₂ [5]. It was established that cell-free extracts of *C. tetanomorphum* could convert glutamate via methylaspartate to mesaconate. The first step in this reaction required a coenzyme of corrinoid nature. The substance was isolated and found to contain two moles of adenine per molecule, one of which seemed to be attached to ribose as in pseudovitamin B₁₂. The other was first supposed to be attached to a double bond in the corrin ring system in a way that greatly influenced the spectrum in the 361 mμ region [4]. A closer examination gave some support for the hypothesis that the second adenine group and an attached unidentified carbon chain are located near the cobalt atom in the space that is partially occupied by the cyano-group in vitamin B₁₂ [22].

Weissbach *et al.* [4] reported that a considerable fraction of the total cobalamin of rabbit liver is present in the coenzyme form. They also suggested that the coenzymes may be the main form of B₁₂ and B₁₂-like compounds, which are catalytically active in the enzymatic reactions of living cells.

In view of these findings it is interesting to note that there is no evidence whatsoever suggesting the presence of adenine in the cobalamin-polypeptide complex. The high ultraviolet absorption around 274 mμ [3] can be accounted for by the content of four tyrosine residues per molecule. The peptide part as examined and described earlier contains nothing but amino acids [3], and the corrinoid is according to the present study pure α-5,6-dimethylbenzimidazolylcobamide. No free adenine could be detected in the starting material or in the products formed by cyanide splitting of the complex.

It was pointed out earlier by the author [2] that vitamin B₁₂ occurs in liver in more than one complex form. As no cyanide treatment was involved in the preparation of the cobalamin peptide, the coenzyme form ought also to be present in some fraction. No assay specific for this substance has been performed as yet in our fraction-

nations. It seems probable that the coenzyme will be strongly adsorbed on the alumina column in step 5 [2] and thus separated from the cobalamin peptide. It is also possible that the coenzyme has been transformed to hydroxocobalamin by the effect of diffuse light in the laboratory and subsequently removed by butanol extraction in step 6.

The absorption spectrum of the pure coenzyme is much different from the peptide complex. In a crude mixture, however, these differences are obscured by absorption from other substances present in the liver which render the spectroscopic detection of the coenzyme difficult.

If Barker is right in his assumption that the cobamide coenzyme is the main form of B₁₂ and B₁₂-like compounds that are catalytically active in the enzymatic reactions of living cells not only in bacteria but also in higher animals, the possibility exists that the cobalamin-polypeptide is a storage form for the vitamin.

The functions attributed to vitamin B₁₂ are so diverse, however, that the present author is inclined to think that corrinoids of other types than the cobamide coenzymes participate as co-factors in several biochemical systems.

SUMMARY

1. The cobalamin-polypeptide from ox liver has been examined and the cobalamin (α -5,6-dimethylbenzimidazolylcobamide) nature of the corrinoid part has been verified.

2. The corrinoid part was isolated as red crystals with the same appearance as cyanocobalamin. It had the following refractive indices: $\alpha = 1.619$, $\beta = 1.647$, $\gamma = 1.659$.

3. The elementary composition of the corrinoid was determined to be: C 56.5, H 6.6, N 14.9, P 2.32, Co 4.39 %.

4. Degradation studies showed that the nucleotide part contained 5,6-dimethylbenzimidazole, ribose, and phosphate in equimolar proportions. The remaining planar structure was identified as factor B (cobinamide).

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A. HEDBOM, *Bound forms of vitamin B₁₂*

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On the synthesis of the isomeric α -methoxythienylacetic acids

By SALO GRONOWITZ and TADEUSZ RAZNIKIEWICZ

It was shown some years ago that α -methoxyphenylacetic acid caused growth response in many dicotyledonous plants [1]. This compound is transported from the upper parts of some plants downwards and is excreted through the roots [2]. It has been shown that treatment of tomato plants with this acid induces the appearance, in the root, of some agent which has a repellent effect on larvae of *Meloidogyne hapla* [3]. Reeve and Pickert [4] have studied the plant regulating properties of 2,4- and 3,4-dichloro- α -methoxyphenylacetic acid as well as of α -methoxydiphenylacetic acid and found that in general these compounds affected dicotyledonous plants in somewhat the same way as 2,4-dichlorophenoxyacetic acid. The growth-regulating properties of many types of organic compounds have been very extensively studied in Uppsala (for reviews, cf. ref. [5] and [6]) and especially the different effects of optical antipodes have been investigated. It is therefore of interest to study also this type of growth-regulating compounds and the differences of their optical antipodes.

In this paper, the preparation of the isomeric α -methoxythienylacetic acids is described. These compounds are interesting in view of the fact that the replacement of a benzene ring by a thiophene ring in biologically active compounds often results in enhanced activity [7].

α -Methoxyphenylacetic acids are simply prepared by methylation of the corresponding mandelic acids with dimethyl sulphate in alkaline solution [8, 4]. However, as the analogous 2-thienylglycolic acid [9] is rather unstable, methylation with dimethyl sulphate leads to α -methoxy-2-thienylacetic acid in rather low yield (30%). Milder conditions for the methylation of α -hydroxyacids were therefore investigated.

Müller and Rundel [10] and Roberts *et al.* [11, 12] have described the alkylation of alcohols with diazomethane, using boron trifluoride-etherate or fluoboric acid as catalyst. The first-mentioned authors report that it is also possible to esterify and methylate α -hydroxyacids, e.g. lactic acid in one step. When we tried to methylate mandelic acid at 0° by this method extensive polymethylene formation occurred and a mixture (1:2) of methyl α -methoxyphenylacetate and methyl mandelate was obtained. By working at -40° it was possible to suppress to some extent the formation of polymethylene and a mixture (2:1) of methyl α -methoxyphenylacetate and methyl mandelate was obtained. The methylated and unmethylated acids can easily be separated after hydrolysis, taking advantage of the relatively low solubility of the sodium salt of the former. An interesting observation is worth mentioning. When the mixture of acids, obtained after hydrolysis, is heated with hot petrol-ether an insoluble residue melting sharply at 89-90° is obtained. This appears to be identical

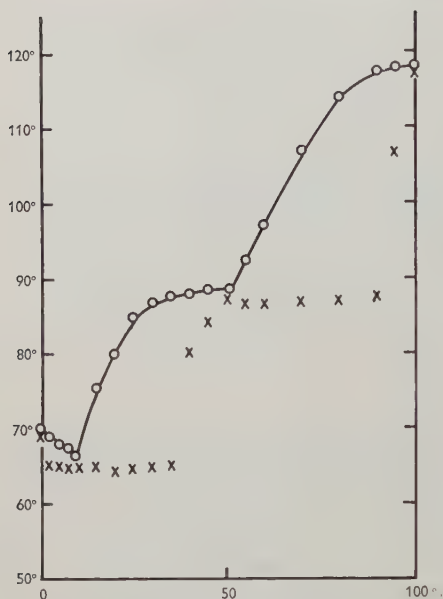


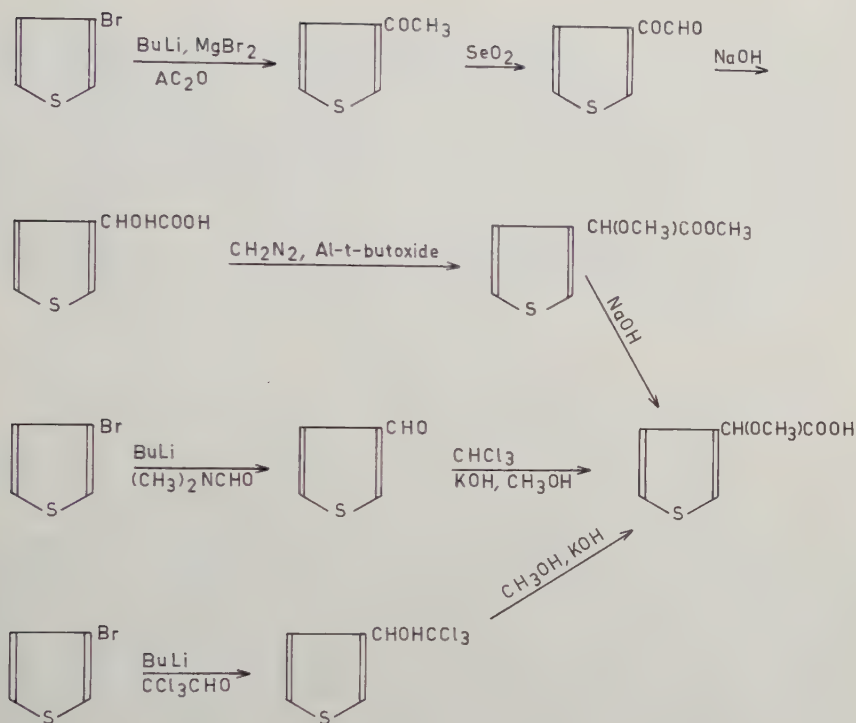
Fig. 1. Mole-% mandelic acid and α -methoxyphenylacetic acid.

with the unidentified product obtained by Hess and Dorner [13] in the reaction of α -chlorophenylacetic acid with sodium methylate. IR-analysis showed it to be an equimolar mixture of mandelic acid and α -methoxyphenylacetic acid. Furthermore, the melting point diagram of the binary system of mandelic acid and α -methoxyphenylacetic acid (Fig. 1) seems to show the pattern associated with an inhomogeneously melting molecular compound [14].

Since the catalyst derived from boron trifluoride proved inadequate, other catalysts were investigated. Meerwein and Hinz [15] demonstrated 30 years ago that not only acids but also compounds such as aluminium alcoholates and ortho esters could catalyze the methylation of alcohols with diazomethane. We found that aluminium *t*-butoxide is an excellent catalyst for the methylation of methyl mandelate and the methyl thienylglycolates. Furthermore, esterification and methylation could be achieved in one step, without any polymethylene formation. The yields of methylated esters when two moles of diazomethane were used were 77–86%.¹ Upon hydrolysis with 2-*N* sodium hydroxide about 60 % over-all yield of methylated acids were obtained.

Since the preparation of the thienylglycolic acids, especially of the 3-isomer, involves a many-step synthesis (cf. reaction scheme) we tried to apply the recent method whereby Reeve and Woods [17] obtained α -methoxyphenylacetic acids directly from the appropriate aldehydes. By reacting the thiophene aldehydes with chloroform and potassium hydroxide in methanol, we obtained 14 % α -methoxy-2-thienylacetic acid from 2-thiophenealdehyde, and 18 % of the 3-isomer from the 3-aldehyde (cf. reaction scheme). The comparable low yields (30 % or less) obtained with this method

¹ A preliminary report of these results was given at "De tredje kemistdagarna i organisk kemi" in Stockholm, June 1960 [16].



are partly due to the reaction between aldehyde and the trichloromethyl aryl carbinol, formed as an intermediate in this reaction [17]. In the reaction with 2-thiophenealdehyde, some 2-thiophenecarboxylic acid was also formed, apparently through a Cannizzaro reaction.

It should be more expedient to prepare the α -methoxythienylacetic acids by reacting the appropriate metal organic compound with chloral, giving directly the trichloromethyl thienyl carbinol intermediate, and obviating the preparation of the aldehydes. This would not entail an additional step, since the metal organic compound has to be prepared in any case as a step in the aldehyde synthesis [18]. On the other hand, by this route one would avoid the complications due to the reaction between the aldehyde and the carbinol intermediate.

Floutz [19] obtained 62 % of trichloromethyl-2-thienylcarbinol from 2-thiophenemagnesium bromide and we obtained 49 % of the 3-isomer through the reaction of 3-thienyllithium [20] with chloral at -70° . The α -methoxy-2-thienylacetic acid was obtained in 46 % yield and the 3-isomer in 72 % by treating the above carbinols with potassium hydroxide in methanol, according to the method used by Weizmann *et al.* [21] for the preparation of α -methoxyphenylacetic acid (they obtained the carbinol intermediate in another way). The three different methods used for the preparation of α -methoxy-3-thienylacetic acid are summarized in the reaction scheme.

α -Methoxy-2-thienylacetic acid exists in several crystalline modifications. A product melting sharply at $59-61^\circ$ is obtained from the melt. Crystallization from a mixture of petrol-ether and benzene gives a product melting during an interval of

64–70° and sometimes even longer. From a slowly evaporating ether-petrol-ether solution, large prismatic crystals can be selected, which have a sharp melting point of 84° and probably represent the optical antipodes. This will be discussed in greater detail in connection with the resolution of this acid into its antipodes [22]. Evidently, the melting point cannot be used as a criterion for purity for this acid. From the IR-spectrum and elementary analyses, however, it is clear that the pure acid had been obtained.

The sodium salts of the α -methoxythienylacetic acids are more soluble than the phenyl analogue and sodium chloride has to be added in order to precipitate them out.

Experimental

Methylation with diazomethane using bortrifluoride-etherate as catalyst

At 0°. To a solution of 7.6 g (0.050 moles) of mandelic acid in 100 ml of dry ether was added drop-wise 100 ml of 0.56-*N* ethereal diazomethane [23]. After 15 min, 0.2 ml of bortrifluoride-etherate [24] was added and then an additional 100 ml of 0.56-*N* diazomethane. The diazomethane colour disappeared with simultaneous formation of polymethylene. The polymethylene (0.49 g, 56.8 %) was filtered off, the ether phase washed with sodium bicarbonate and water, dried and fractionated, yielding 6.5 g of a product, b.p. 126–130°/11 mm Hg, which was shown by quantitative IR-analysis to consist of 63 % methyl mandelate and 36 % of methyl α -methoxyphenylacetate. Increasing the amount of bortrifluoride-etherate to 0.6 ml gave 0.67 g (77 %) of polymethylene and 6.5 g of an ester mixture consisting of 70 % methyl mandelate and 30 % methyl α -methoxyphenylacetate.

At –40°. When the reaction was carried out at –40° using 1.5 ml of bortrifluoride-etherate, 0.12 g (14 %) only of polymethylene was obtained. Work-up in the same manner as above yielded 6.8 g of methylated product, b.p. 127–135°/13 mm Hg, consisting of 36 % methyl mandelate and 63 % of methyl α -methoxyphenylacetate.

The ester mixture was heated on a steam-bath with 42 ml of 2-*N* sodium hydroxide until a homogeneous solution was obtained. It was acidified, extracted with ether and the solvent driven off, yielding 5.8 g of crude acid, consisting of 64 % of α -methoxyphenylacetic acid and 36 % of mandelic acid. Treatment with hot petrol-ether (b.p. 40–60°) yielded a residue of 4.1 g, m.p. 89–90°. IR-spectrum and equiv. wt. (159.6) showed it to be an equimolar mixture of mandelic acid and α -methoxyphenylacetic acid. From the petrol-ether solution, 1.2 g of pure α -methoxyphenylacetic acid, m.p. 70–72° was obtained.

*Methylation with diazomethane using aluminium *t*-butoxide as catalyst*

Methyl α -methoxyphenylacetate. To 38.1 g (0.251 moles) of mandelic acid was added drop-wise 450 ml of 0.625-*N* ethereal diazomethane. After 15 min, 2.5 g (0.010 moles) of aluminium *t*-butoxide [25] was added followed by 380 ml of diazomethane solution. After standing over night, the solution was colourless and no polymethylene had separated. It was washed with 200 ml of *N*-hydrochloric acid and water, dried over magnesium sulphate and distilled, yielding 36.3 g (77 %) of methyl α -methoxyphenyl-

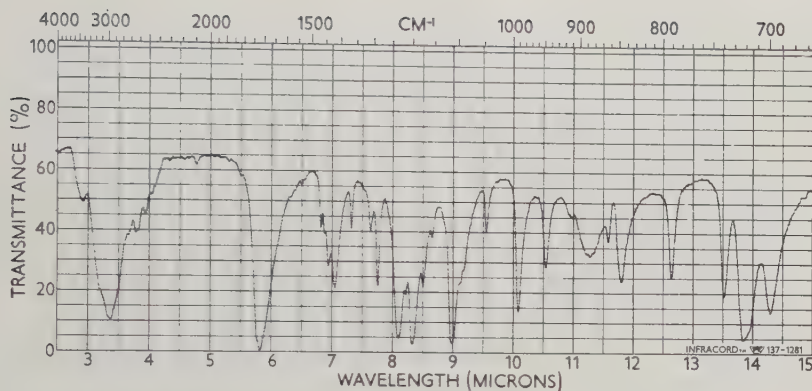


Fig. 2. IR-spectrum of α -methoxy-2-thienylacetic acid in KBr.

acetate, b.p. 117–118°/9 mm Hg, $n_D^{20} = 1.5063$. (Literature value [26], b.p. 116–117°/10 mm Hg, $n_D^{20} = 1.5110$.)

α -Methoxyphenylacetic acid. 36 g (0.200 moles) of the ester was heated on a steam-bath with 250 ml of 2-*N* sodium hydroxide until a clear solution was obtained. Upon cooling, 33.8 g of the sodium salt was filtered off and washed with a little acetone. From the mother-liquor, additional 4.4 g of sodium salt was obtained upon evaporation to half the volume. 38.2 g of the sodium salt was suspended in 150 ml of water and acidified with 120 ml of 4-*N* hydrochloric acid. Extraction of the mixture with ether yielded 31.1 g (94 %) of crude acid. Recrystallization from 950 ml of ligroin (b.p. 80–100°). Yielded 25.3 g (76 %) of pure α -methoxyphenylacetic acid, m.p. 69–70°. (Literature value [8], m.p. 71°.)

Methyl α -methoxy-2-thienyl acetate. From 37 g (0.234 moles) of 2-thienylglycolic acid [9] in 200 ml of ether, 3.2 g aluminium *t*-butoxide and 775 ml of 0.62-*N* ethereal diazomethane were obtained, as described above, 37 g (85 %) of methyl α -methoxy-2-thienylacetate, b.p. 127°/12 mm Hg $n_D^{20} = 1.5172$.

α -Methoxy-2-thienylacetic acid. Method I. 36.5 g (0.196 moles) of the ester was hydrolyzed as above with 9.7 g of sodium hydroxide in 120 ml of water. 38 g of sodium chloride was then added, and upon cooling 40 g of the sodium salt separated and was filtered off. After acidification of the salt with 225 ml of *N*-hydrochloric acid and extraction with ether, 30 g (89 %) of crude acid was obtained. Recrystallization from 1500 ml of a mixture of petrol-ether and benzene (2:1) yielded 25.5 g (76 %) of pure colourless needles of α -methoxy-2-thienylacetic acid, m.p. 64–70°. IR-spectrum, Fig. 2.

Anal. $C_7H_8O_3S$ (172.2)

Calc. Equiv. wt. 172.2 C 48.82 H 4.68 S 18.62

Found Equiv. wt. 173.0 C 49.07 H 4.72 S 18.50

Methyl α -methoxy-3-thienylacetate. 17.8 g (0.113 moles) of 3-thienylglycolic acid [9] in 150 ml of ether, 1.5 g of aluminium *t*-butoxide and 380 ml of 0.65-*N* ethereal diazomethane solution yielded, as described above, 18.1 g (86 %) of methyl α -methoxy-3-thienylacetate, b.p. 128.5–129°/14 mm Hg, $n_D^{20} = 1.5168$.

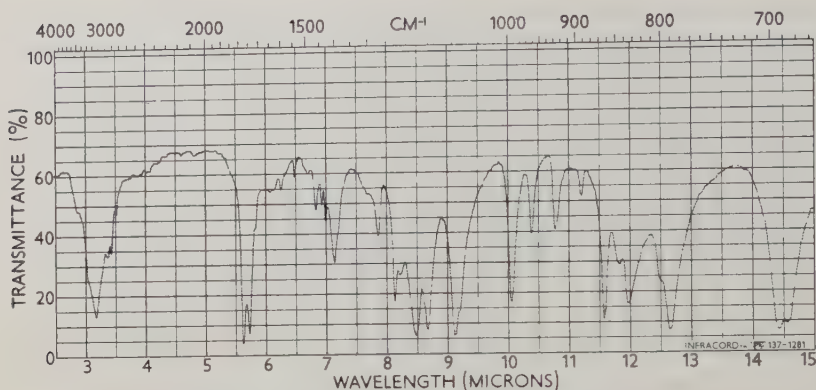


Fig. 3. IR-spectrum of α -methoxy-3-thienylacetic acid in KBr.

α -Methoxy-3-thienylacetic acid. Method I. 16.7 g (0.0897 moles) of the above ester was hydrolyzed with 65 ml of 2-N sodium hydroxide. After adding 22 g of sodium chloride and cooling, 21.5 g of the sodium salt separated. Acidification and extraction with ether yielded 13.3 g (86 %) of crude acid. Crystallization from a mixture of petrol-ether-benzene (2:1) yielded 12.2 g (79 %) of pure colourless prisms of α -methoxy-3-thienylacetic acid, m.p. 56–59°. IR-spectrum, Fig. 3.

Anal. $C_7H_8O_3S$ (172.2)

Calc. Equiv. wt. 172.2 C 48.82 H 4.68 S 18.62

Found Equiv. wt. 173.2 C 49.08 H 4.71 S 18.56

α -Methoxy-thienylacetic acids via trichloromethyl thienyl carbinols

α -Methoxy-2-thienylacetic acid. Method II. To a solution of 30 g (0.268 moles) of 2-thiophenealdehyde [27] and 47.7 g of chloroform (0.400 moles) in 70 ml dry methanol was added dropwise at 45° 360 ml of 3.7-N methanolic potassium hydroxide. The mixture was kept at this temperature over night, heated for 1.5 h at 70°, cooled and the precipitated potassium chloride (81 g) filtered off. The methanol was removed *in vacuo*. The residue was dissolved in 125 ml of water, 38 g of sodium chloride was added and the precipitated sodium salt was filtered off and washed with acetone. Work-up as above yielded 6.7 g (15 %) of α -methoxy-2-thienylacetic acid, m.p. 59–70°. The IR-spectrum was identical to that of the product obtained by method I.

α -Methoxy-3-thienylacetic acid. Method II. To a solution of 48 g (0.428 moles) of 3-thiophenealdehyde [28] and 76.5 g (0.641 moles) of chloroform in 85 ml of methanol was added a solution of 140 g of potassium hydroxide in 340 ml of methanol and kept at 45° over night. Potassium chloride was filtered off and the filtrate acidified with 6-N hydrochloric acid to about pH 3. Upon addition of sodium chloride solution, no sparingly soluble acid salt separated, so the mixture was strongly acidified and the product extracted with ether. The combined ether phases were extracted with sodium bicarbonate solution (500 ml) but since addition of sodium chloride caused no precipitation, the solution was acidified and extracted with ether. The ether was evaporated and the residue dissolved in a solution of 9 g of sodium hydroxide in 85 ml

of water. Addition of sodium chloride precipitated the sodium salt, which was filtered off, acidified and extracted with ether. Evaporation yielded 15.0 g (20 %) of acid which was recrystallized from petrol-ether-benzene (2:1) yielding 13.1 g (18 %) of pure α -methoxy-3-thienylacetic acid, m.p. 56–59°. The IR-spectrum was identical with that of the product obtained by method I.

α -Methoxy-2-thienylacetic acid. Method III. To a solution of 50 g (0.216 moles) of trichloromethyl 2-thienylcarbinol [19] in 80 ml of dry methanol was added drop-wise at 45° 238 ml of 3.7-N methanolic potassium hydroxide. The mixture was then refluxed for one hour, cooled and the potassium chloride (45.8 g) filtered off. The clear solution was evaporated *in vacuo*, the residue (64 g) dissolved in 127 ml of hot water and 37 g of sodium chloride added. Upon cooling, 41.3 g of sodium salt separated, which was filtered off and carefully washed several times with acetone in order to remove oily impurities. The usual work-up of the salt yielded 18.5 g (50 %) of acid which upon recrystallization yielded 17.3 g (47 %) of pure α -methoxy-2-thienylacetic acid, m.p. 59–70°. Equiv. wt. 173.4. The IR-spectrum was identical with that of the product prepared according to methods I and II.

Trichloromethyl 3-thienylcarbinol. To a solution of 3-thienyllithium [20] prepared in the usual manner at –70° from 514 ml of 1.07-N *n*-butyllithium and 82.5 g (0.506 moles) of 3-bromothiophene [29] in 85 ml of ether was added drop-wise 74.7 g (0.507 moles) of chloral in 150 ml of ether. After 15 min, the cooling bath was removed and when the temperature had risen to about 0°, the mixture was poured into ice-water and 65 ml of acetic acid was added. The ether phase was separated and the water phase extracted with ether. The combined ether phases were washed with sodium carbonate and water, dried and distilled, giving 49 % of crude isomer-free trichloromethyl 3-thienylcarbinol, b.p. 137–152°/9 mm Hg, n_D^{20} = 1.5832, which was used without purification in the next step.

α -Methoxy-3-thienylacetic acid. Method III. This was prepared like the 2-isomer, from 35.0 g (0.151 moles) of trichloromethyl 3-thienylcarbinol in 75 ml of methanol and 150 ml of 4.1-N methanolic potassium hydroxide. 18.8 g (72 %) of pure α -methoxy-3-thienylacetic acid, m.p. 56–59° was obtained, identical with the product obtained according to methods I and II.

Methylation with dimethyl sulphate

α -Methoxy-2-thienylacetic acid. Method IV. To a solution of 9.6 g (0.0607 moles) of 2-thienylglycolic acid and 31.8 g of sodium hydroxide in 105 ml of water was added drop-wise 41.6 g (0.330 moles) of redistilled dimethyl sulphate at 45–50°. 9.6 g of product was filtered off after cooling and dissolved in 70 ml of hot water. 17.3 g of sodium chloride was added, and the solution cooled, whereupon 4.9 g of the sodium salt crystallized out. 3.1 g (30 %) of pure α -methoxy-2-thienylacetic acid, m.p. 60–62° was obtained by treating it in the usual manner.

The melting points and the melting point diagram were determined in a hot stage microscope. The IR-spectra were recorded on a Perkin-Elmer Infracord spectrophotometer. The peaks at 2.88μ (3470 cm^{-1}) of methyl mandelate and at 9.84μ (1016 cm^{-1}) of methyl α -methoxymandelate were used for the analysis of the mixtures obtained. Calibration curves were constructed from mixtures of known compositions.

SUMMARY

The isomeric α -methoxythienylacetic acids have been prepared:

- (a) by methylation of the thienylglycolic acids with dimethyl sulphate (2-isomer only);
- (b) by methylation with diazomethane, using aluminium *t*-butoxide as a catalyst;
- (c) through the base-catalyzed condensation of the thiophenealdehydes with chloroform and methanol; and
- (d) through the reaction of the trichloromethyl thienylcarbinols with methanolic potassium hydroxide. The last-mentioned method is considered to be the best.

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Chemical Institute, University of Uppsala, Uppsala, Sweden. January 1961.

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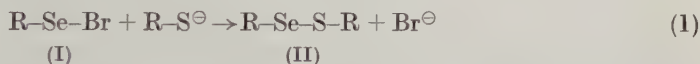
Some experiments with aliphatic selenenylbromides and thiolseLENENATES

By GÖRAN BERGSON and GUNBRITT NORDSTRÖM

With 6 figures in the text

The aim of the present investigation is to study some reactions of aliphatic selenenylbromides (I) which might involve the formation of a sulphur-selenium bond, and to explore the ultra-violet spectrum of some simple thiolseLENENATES (II).

The reaction between a thiolate ion and a selenenylbromide should give a thiolseLENENATE in a nucleophilic substitution at the selenium atom:



Since selenenylbromides are highly reactive compounds which readily undergo solvolysis in hydroxylic solvents [1], the reaction (1) must be carried out in an inert solvent such as benzene, chloroform or cyclohexane. This tendency to solvolysis might also mean that thiolseLENENATES could perhaps be formed from thioles and selenenylbromides:



The reactions (1) and (2) represent the two fundamental mechanisms by which a halogen atom in a molecule may be substituted by another nucleophilic group. The displacement of bromine in selenenylbromides by the R-S- group constitutes a special example of the substitution of halogen by R-S- or R-Se- groups now being studied at this institute. Other halogen compounds under investigation are triphenylmethylchloride and chloroacetone.

A reaction of this type, leading to a thiolseLENENATE has been selected for the purpose of an experimental ultraviolet spectroscopic investigation of the electronic structure of the sulphur-selenium bond. It has been suggested that an unsymmetrical disulphide (thiolsulphenate) might give rise to *two* absorption bands, due to the possibility of electron excitation from both sulphur atoms [2]. The theory for the ultraviolet absorption of disulphides developed by Bergson [3, 4, 5], however, suggests that there is no fundamental difference in this respect between symmetrical and unsymmetrical disulphides; hence if two bands are not observed for a symmetrical molecule it is very unlikely that they would be observed for the unsymmetrical one. Some unsymmetrical halogen substituted non-cyclic disulphides have been investigated [6], but they were found to be unsuitable for ultra-violet study since they did not show any pronounced absorption peaks. Unsymmetrical carboxylic substituted 1,2-

dithiolane derivatives were found to give two UV-peaks which were interpreted as being due to the asymmetry of the S-S bond [2], but this interpretation has been questioned [7]. The thiolselenenates may be imagined as highly unsymmetrical disulphides (or diselenides) and an ultra-violet investigation of such compounds therefore seemed worth while.

Previous studies in the aromatic series. Aliphatic thiolselenenates do not seem to have been described previously. A large number of aromatic analogues have, however, been synthesized by Rheinboldt and Giesbrecht [8]. Their methods of synthesis depended on the reactions between aromatic selenenylbromides and aromatic or aliphatic thioles, or between sulphenylhalogenides and selenoles, in ether, chloroform, benzene or toluene. These authors also report that thiolate or selenolate ions may be used in the reaction. The selenenylbromides used were prepared from the corresponding selenenylcyanides and bromine. In all the reactions studied the yields of thiolselenenates were very good.

Nakazaki [9] has drawn attention to the similarity of the -CN group to the halogens. He treated some aromatic selenenylcyanides with thioles and obtained good yields of thiolselenenates according to the reaction formula:



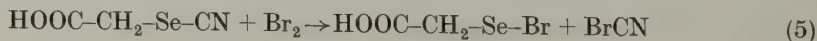
The formation of selenenylbromides

There seem to be two methods which might be convenient for the formation of selenenylbromides, viz. the action of bromine on either diselenides or selenenylcyanides [10]. As an example of the first method, we treated diethyldiselenide with the calculated weight of bromine using chloroform or cyclohexane as solvents:



The reaction proceeded rapidly and a fairly stable, dark red solution resulted. When the solution was stored for two days in the dark no change in the absorption spectrum could be noted. Longer exposure to daylight, however, induced slow decomposition. In the preparations some tribromide ($\text{C}_2\text{H}_5\text{SeBr}_3$) could be detected due to local excess of bromine; this, however, rapidly disappeared on mixing. The absorption spectrum of ethylselenenylbromide in cyclohexane is given in Fig. 1. The bromide has two characteristic absorption peaks at 268 and 440 $m\mu$ in chloroform, and at 275 and 448 $m\mu$ in cyclohexane. Diethyldiselenide has an absorption maximum at 312 $m\mu$ [4] and bromine, as we have found, at 272 and 406 $m\mu$ (in chloroform). It should be noted that the ultra-violet absorption changes proceed rapidly until the curves in Fig. 1 result; this and the fact that the slightly soluble tribromide rapidly dissolves on mixing as just mentioned, together with the above figures, constitute a full proof for the quantitative formation of ethylselenenylbromide in these experiments. An independent proof for the quantitative formation of a selenenylbromide in the reaction between a diselenide and an equimolar amount of bromine is found in the polarimetric investigation of the reaction between optically active diselen- α -di-propionic acid and bromine studied by Fredga [23].

The reaction between bromine and a selenenylcyanide was tested using selenocyan acetic acid in chloroform:



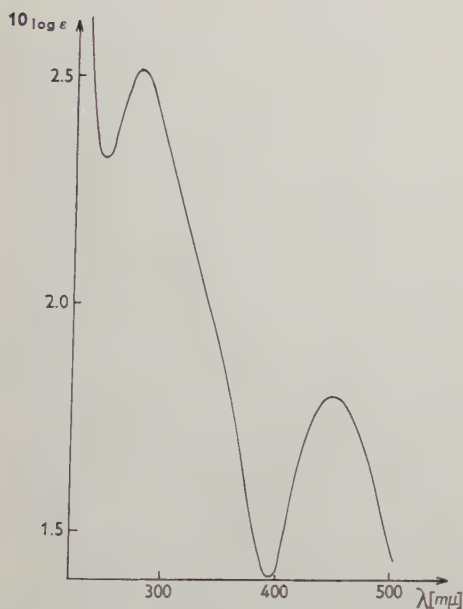


Fig. 1. Ultra-violet spectrum of $\text{C}_2\text{H}_5\text{SeBr}$ in cyclohexane.

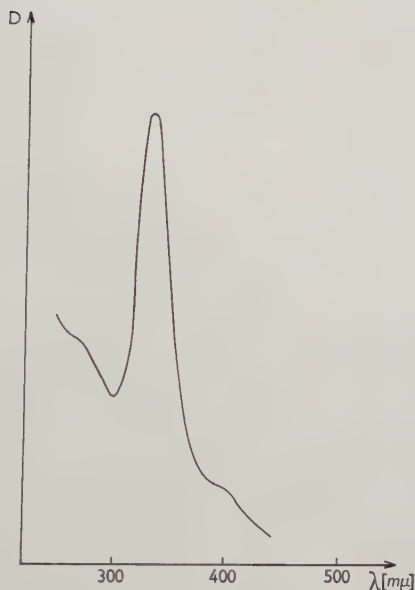
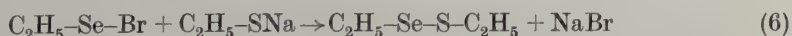


Fig. 2. Ultra-violet spectrum of $\text{HOOC-CH}_2\text{SeBr}$ in chloroform.

Due to the slight solubility of selenocyan acetic acid the reaction was only complete after the reaction mixture had been shaken for some hours. The solvent and bromocyan were removed in vacuum at room temperature and a dark red oil resulted. The optical density of a rapidly prepared chloroform solution of the substance is given in Fig. 2. Although carboxylic groups are known to be very slightly nucleophilic the possibility of self-solvolysis of this selenenylbromide cannot be ruled out. The solvolysis of a selenenylbromide group by a carboxylic group must, however, be very slow, since no change in the optical density of a solution of ethylselenenylbromide in chloroform containing excess of acetic acid could be detected, even after several hours. Nor could any changes be noted in the solution of the selenenylbromide prepared from selenocyanacetic acid upon addition of acetic acid.

Reaction between selenenylbromides and thiolate ions

The solutions of ethylselenenylbromide in chloroform or cyclohexane were shaken with solid sodium ethylthiolate for a few hours. The characteristic colour of the selenenylbromide disappeared and sodium bromide was formed quantitatively according to the formula:



These observations alone do not constitute a full proof for the formation of a sulphur-selenium band. In view of the instability of unsymmetrical disulphides [11] it is

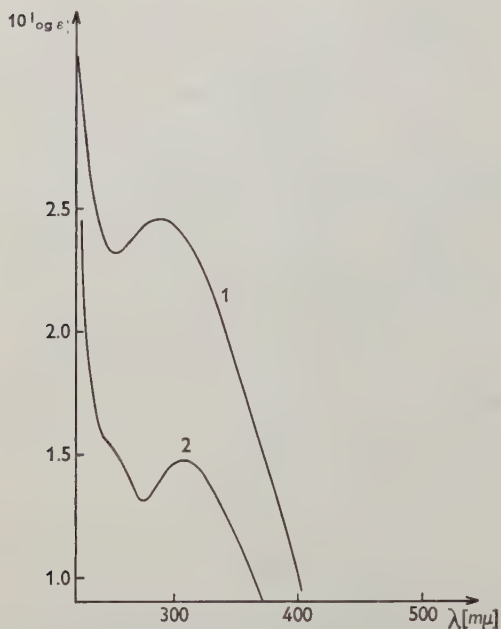


Fig. 3. Ultra-violet spectrum of $C_2H_5SeSC_2H_5$ (1) and its decomposition products (2).

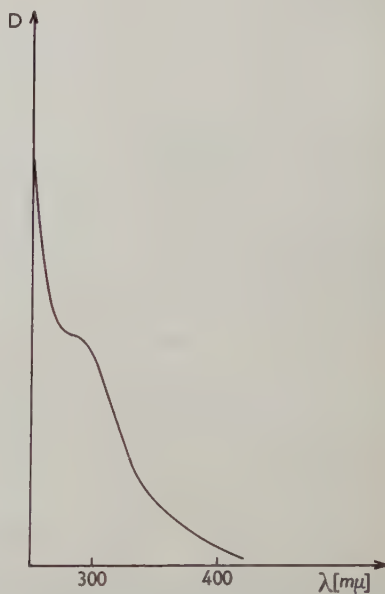


Fig. 4. Ultra-violet spectrum probably due to the presence of $C_2H_5SSeCH_2-COOH$.

possible that the ethylthiolselenenate might rapidly decompose into diethyldisulphide and diethyldiselenide:



In the opinion of the authors, if the formation of a selenium-selenium and a sulphur-sulphur bond can be ruled out, then this fact, together with the quantitative yield of sodium bromide, proves that a sulphur-selenium bond is formed. The diethyldisulphide and diethyldiselenide have absorption peaks at 251 and 312 $m\mu$ respectively [12] and the solution resulting from the reaction between ethylselenenylbromide and sodium ethylthiolate has no such peaks. It has instead a pronounced absorption maximum at 285 $m\mu$ as shown in fig. 3. This peak must therefore be due to the presence of ethylthiolselenenate. Thus the primary aim of these experiments, namely the formation of a sulphur-selenium bond and the observation of an ultra-violet spectrum of an aliphatic thiolselenenate, has been achieved.

As might be expected, the thiolselenenate is fairly unstable. The removal of the solvent in vacuum resulted in decomposition into disulphide and diselenide according to reaction formula (7). The yellow oil produced by decomposition was again dissolved and the absorption spectrum of this solution clearly shows the presence of disulphide and diselenide (Fig. 3). Extremely mild treatment in the dark might perhaps give the thiolselenenate in pure form, but this lies beyond the scope of the present investigation. In view of the behaviour of the corresponding disulphides and diselenides, carboxylic substituted thiolselenenates may be expected to be less suited for spectro-

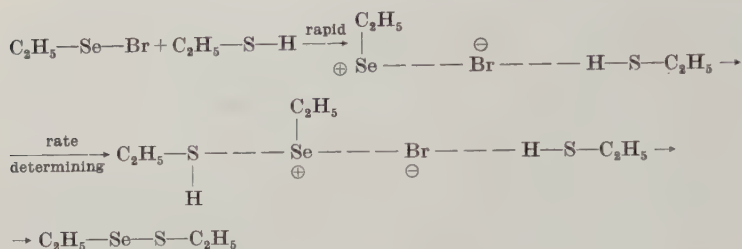
scopic study. Nevertheless, some preliminary experiments were done with selenenylbromide acetic acid. In the reaction of this compound with thiolate ions or with thioles, precipitation of elemental selenium was frequently observed. However, when the thiolate (sodium ethylthiolate) was present in excess a solution showing an absorption curve with a shoulder at about $280\text{ m}\mu$ was formed (Fig. 4). This effect may be due to the presence of a sulphur-selenium bond.

The presence of the carboxylic group in the close vicinity of the selenenylbromide group evidently has an adverse effect on the stability of the carbon-selenium bond. In order to determine the extent of the fission of the carbon-selenium bond in selenenylbromide acetic acid, the reactions of the compound with other nucleophilic reagents were examined. First of all, we found that acetate ions (in contrast to acetic acid) caused rapid precipitation of selenium. The relative acid strengths of carboxylic acids and thiols therefore show why the substitution of bromine by the thiolate ion is completely unsuccessful unless the thiolate is present in large excess. We then found that precipitation of selenium from selenenylbromide acetic acid was caused by a variety of nucleophilic reagents, e.g. iodide ions, ethanol, ethylthiol, thioglycolic acid and water. No reaction was observed with weak nucleophilic reagents such as chloride ions and acetic acid (see above). Although, as mentioned above, excess thiolate seems to give a sulphur-selenium bond, there is also the possibility that thiolate ions may cause fission of the carbon-selenium bond. Some selenium was always found even in these experiments, and evidently there are at least two competing reactions in operation. However, it was found that ethylselenenylbromide reacts rapidly with all the nucleophilic reagents listed above (except of course chloride ions and acetic acid). This is shown by the disappearance of the characteristic colour without any selenium being formed. Hence it is clearly the carboxylic group in selenenylbromide acetic acid which is responsible for the fission of the carbon-selenium bond. The preparation of a carboxylic substituted thiol-selenenate might be achieved using the reaction between, e.g., ethylselenenylbromide and thioglycolic acid. It is perhaps not desirable to speculate about the mechanism of the fission of the carbon-selenium bond. However, two possibilities suggest themselves, viz. that the inductive effects of the carboxylic group either causes a weakening of the carbon-selenium bond in the transition state of a substitution at selenium, or induces a competing reaction involving attack at carbon which would easily result in the formation of elemental selenium.

Reaction between selenenylbromides and thioles

The solvolysis of ethylselenenylbromide with ethyl thiol in cyclohexane proceeds very rapidly. The dark red colour of the selenenylbromide disappeared at once when the calculated quantity of thiol had been added, and hydrogen bromide was evolved. A stream of nitrogen was passed through the solution and the resulting yellow solution showed an ultra-violet absorption curve identical with that for ethyl thiol-selenenate prepared from ethylselenenylbromide and sodium ethyl thiolate.

The high speed of the reaction seems to make it difficult to obtain any kinetic evidence for the reaction mechanism. In view of recent investigations on the solvolysis of the carbon-halogen bond [13] a "push-pull" mechanism seems plausible, although the importance of the hydrogen bonding may be slight in our case when the solvating agent is a thiol:



Discussion of the ultra-violet spectrum

It has been well established that simple non-cyclic disulphides (thiolsulphenates) have an absorption band with maximum at about $250 \text{ m}\mu$ [14] while the corresponding diselenide peak is at about $310 \text{ m}\mu$ [4]. The cause of this absorption has been interpreted by Bergson [3] in terms of the excitation of an electron from the antibonding π -orbital to the antibonding σ -orbital of the S—S bond. If the dihedral angle of the C—S—C group (i.e. the angle between the planes CSS and SSC) is exactly 90° and the atomic orbitals on the sulphur atoms are pure s - and p -orbitals, the bonding and antibonding π -orbitals will degenerate to pure atomic p -orbitals; i.e. the electron pairs are completely localized to the corresponding atoms (Fig. 5). Under these boundary conditions there should be two coinciding excitations for a symmetrical disulphide or diselenide, but for unsymmetrical compounds and thiolselelenates two non-degenerate excitations should be possible. However, in reality these boundary conditions are never fulfilled. Even if no hybridization is assumed, the electronic picture for symmetrical and unsymmetrical molecules tends to become very similar as the dihedral angle departs from 90° ; which is evident from Fig. 5. If the dihedral angle deviates only a little from 90° , as in a non-cyclic compound, the two possible absorption peaks would be very difficult to observe. This is because of the broad character of the absorption bands which to a large extent must be due to the thermal torsional motion about the S—S, Se—Se or Se—S bond. In a five-membered ring system, e.g. 1,2-dithiolane, the dihedral angle is about 27° [15] and a large difference in the excitation energies from the bonding and antibonding π -orbitals must result. Furthermore, the thermal torsional motion must be greatly suppressed in such a rigid ring system. The complication that arises here, however, is that the strong absorption at wavelengths shorter than $250 \text{ m}\mu$, which is always found in sulphur and selenium compounds, probably overlaps any absorption band due to transition from the bonding π -orbital. An intermediate case would be found in a six-membered ring where the dihedral angle is about 60° [16]. An absorption band which has previously been overlooked can be detected in the absorption curve for 1,2-dithiane (shoulder at $240\text{--}245 \text{ m}\mu$) recorded by Calvin and co-workers [17] and for 3,3,6,6-tetramethyl-1,2-dithiane (peak at $243 \text{ m}\mu$) recorded by Claeson [18]. These absorption bands may be due to a transition from the bonding π -orbital although the assignment must not be regarded as quite certain.

It only remains to be added that the similarity in the electronic structure between symmetrical and unsymmetrical molecules becomes still more pronounced if the lone-pair electrons occupy hybridized orbitals. A calculation of the orbital energies using sp^3 -atomic orbitals in a symmetrical disulphide has been made by Bergson [5] and the result is given schematically in Fig. 6. It shows that the bonding and antibond-

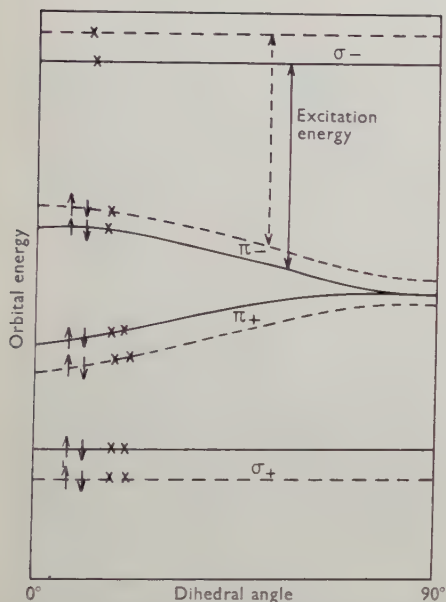


Fig. 5. Energies of bonding (+) and antibonding (—) orbitals in symmetrical disulphides (—) and in unsymmetrical molecules (---). ↑ denotes electrons in the ground state and × in the first excited state. The molecular orbitals are formed by linear combination of pure atomic orbitals.

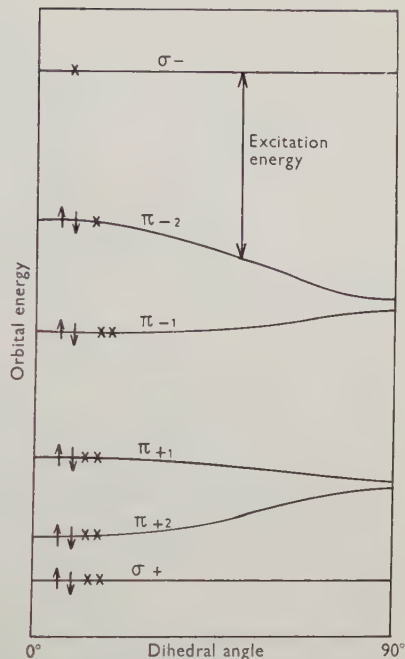


Fig. 6. Orbital energies in a symmetrical disulphide where the molecular orbitals have been formed by linear combination of sp^3 -hybridized atomic orbitals.

ing π -orbitals do not coincide here. In fact, a certain amount of hybridization must also be assumed since the *bond angle* at the sulphur or selenium atom is always greater than 90° [19].

The conclusion to which all these arguments lead (hybridization, thermal torsional motion in non-cyclic molecules and large deviation from 90° in the dihedral angle in cyclic compounds) is that there is no fundamental difference between symmetrical and unsymmetrical molecules, the difference being only quantitative and not qualitative. We therefore think that our experimental observation (Fig. 3) that ethyl thiol-selenenate gives an absorption curve very similar to those for diethyldisulphide and diethyldiselenide is an important support for the theoretical approach being made in the understanding of the phenomena connected with the S-S, Se-Se and Se-S bonds. Our present work on the sulphur-selenium bond is concerned with an examination of five-membered ring systems in connection with studies on compounds related to thioctic acid, and some preliminary findings have been reported [20]. We found that 3-(1-thia-2-selena-cyclopentane)- δ -valeric acid has only a single absorption maximum above $230\text{ m}\mu$ at $382\text{ m}\mu$. The corresponding peak in the analogous sulphur compound (thioctic acid) lies at $330\text{ m}\mu$ and in selenoctic acid at $442\text{ m}\mu$ [4].

Experimental

Diethyldiselenide was prepared from diethylsulphate and sodiumdiselenide according to [21]. B.p.₂₁: 85°. $n_{20}^D = 1.5831$.

Selenocyan acetic acid was prepared according to [22] from chloroacetic acid and potassium selenocyanate. Our preparation was contaminated with a small amount of diselen-diacetic acid which, however, cannot interfere with our result. M.p. 76–81°.

Ethyl selenenylbromide. 0.0961 g (4.5×10^{-4} moles) of diethyldiselenide was dissolved in chloroform. 33.8 ml (4.5×10^{-4} moles) of a 1.316×10^{-3} molar solution of bromine was added to this solution. Total volume 93.8 ml. Concentration of selenenylbromide: 9.48×10^{-3} molar. This solution was used as a stock solution in our experiments.

A 9.40×10^{-3} molar solution of ethyl selenenylbromide in cyclohexane was prepared in a similar way.

Ethyl thiolselelenate. (a) Solid sodium ethylthiolate, prepared from ethylthiol and sodium ethoxide in ethanol followed by evaporation of the solvent, was added to 50 ml of the ethyl selenenylbromide solution and the mixture was shaken until the colour of the selenenylbromide had disappeared. The sodium bromide was removed by filtration and washed with known amounts of solvent. The concentration of ethyl thiolselelenate in chloroform was 8.62×10^{-3} molar and in cyclohexane 9.04×10^{-3} molar.

(b) 8.68 ml of 0.2141 molar bromine solution was added to 10 ml of a 0.1859 molar solution of diethyldiselenide in cyclohexane. The resulting solution was diluted to 25 ml. 10 ml of this 0.1487 molar solution of ethyl selenenylbromide 6.93 ml of a 0.2145 molar solution of ethylthiol was added and the total volume made up to 25 ml after removal of hydrogen bromide by passing nitrogen through the solution. This 5.95×10^{-2} molar solution of ethyl thiolselelenate showed an absorption spectrum identical with that prepared in cyclohexane according to (a).

SUMMARY

1. Reaction between bromine and diethyldiselenide or selenocyan acetic acid in chloroform or cyclohexane gives the corresponding selenenylbromides. The ultra-violet absorptions of these compounds have been given.

2. Substitution of the bromine atom in ethyl selenenylbromide by acetate, iodide and thiolate ions and by water, ethanol and thioles takes place readily, without any cleavage of the carbon-selenium bond. The substitution performed by sodium ethylthiolate and ethanethiol results in the formation of ethyl thiolselelenate. This compound is unstable and decomposes into diethyldisulphide and diethyldiselenide.

3. Fission of the carbon-selenium bond in selenenylbromide acetic acid occurs readily when it is treated with the nucleophilic reagents listed under 2.

4. The ultra-violet absorption spectrum of ethyl thiolselelenate shows an absorption peak at 285 mμ. A discussion of this spectrum in relation to the spectra of disulphides and diselenides is given. The similarities in the electronic structures of symmetrical and unsymmetrical molecules are pointed out.

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Uppsala 1961. Almqvist & Wiksells Boktryckeri AB

Sensitized photochemical depolymerization of cellulose nitrate in solution

By STIG CLAESSON, GÖSTA PALM and GUNNAR WETTERMARK

With 4 figures in the text

In a previous investigation solutions of cellulose nitrate were irradiated with light of wavelength $253.7\text{ m}\mu$ [1]. The depolymerization was followed viscosimetrically, and was found to obey the theory for a random process, with a quantum yield of 0.01–0.02 for the chain scissions.

It is now desirable to obtain information on the magnitude of this degradation when the energy of the light is varied. The light could then be absorbed by the nitrocellulose or by another molecule which acts as a sensitizer for the degradation. The effectivity of a sensitized degradation is normally quite low. A study of these phenomena thus required light of high intensity in order to get measurable effects, keeping the exposure times within a reasonable range.

Ultraviolet light with a high spectral purity, and high intensity can only simultaneously be achieved by employing a monochromator of great luminosity. This investigation was thus made possible by the use of a powerful waterprism monochromator [2]. From preliminary tests, it was found that the addition of many substances markedly increased the degradation rate, when nitrocellulose was exposed to ultraviolet light. For the quantitative measurements a system of nitrocellulose, methanol and β -naphthylamine was chosen.

A commercial sample of partially nitrated cellulose (Kollodium bomull, Bofors Nobelkrut VF 500) was fractionated in an acetone water system. A middle fraction, having an intrinsic viscosity in methanol of 4.14, was used in the experiments. The nitrogen content of the fraction was 12.12 %. Methanol, *pro analysi* (LKB) was dried with Drierite and distilled, β -naphthylamine, *pure* (The Coleman and Bell Co., Norwood, O., U.S.A.) was recrystallized five times from ethanol in complete darkness. It was dried in air at room temperature.

Irradiations were performed on two sets of solutions. One set contained only nitrocellulose in methanol, and the other, in addition, had β -naphthylamine. Before the irradiations a stream of nitrogen was passed through the solution. Exposures were made using the lines with the wavelengths 302, 334, and $365\text{ m}\mu$ in the spectrum from a high pressure mercury lamp (Phillips SP 500 W). The lines were isolated using a waterprism monochromator [2]. The widths of the transmitted bands are 2.8, 4.0 and $5.1\text{ m}\mu$ respectively. Irradiations were made in cylindrical quartz vessels (inner diameter 12 mm) with ground quartz stoppers.

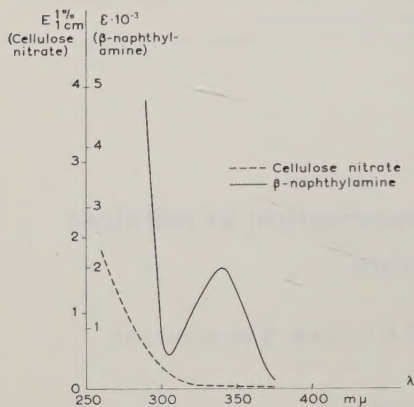


Fig. 1.

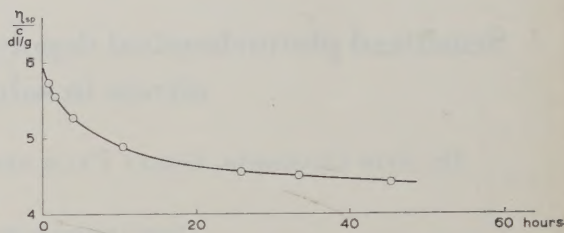


Fig. 2.

Fig. 1. Absorption spectra for cellulose nitrate and β -naphthylamine. Beer's law is used in the form $^{10}\log(I_0/I) = E_{1\text{cm}}^{1\%} \cdot c \cdot l$ and $^{10}\log(I_0/I) = \epsilon \cdot c \cdot l$ for nitrocellulose and β -naphthylamine respectively. $E_{1\text{cm}}^{1\%}$ is the extinction coefficient for $c = 1\%$ and $l = 1$ cm and ϵ is the extinction coefficient for $c = 1$ mole/l and $l = 1$ cm.

Fig. 2. η_{sp}/c versus time after exposure (the post-irradiation effect), for a solution of cellulose nitrate in methanol. An incident dose of 10.9×10^{18} hr at wavelength $302 \text{ m}\mu$ was given to the sample. The viscosities were measured at a concentration of $0.2695 \text{ g}/100 \text{ ml}$.

Two equivalent vessels are mounted on the same axis and rotated during exposure. The volume of the two cuvettes is 3.50 and 3.29 ml respectively. The light is introduced through a $1 \times 14 \text{ mm}$ slit parallel to the axis of the cuvettes. A thorough description of the mounting is found in the article on the monochromator [2]. One vessel contains a nitrocellulose solution and the other an actinometer solution. The vessels are then alternately introduced into the beam of light in order to compensate for changes in the intensity of the light that may occur.

The light doses were determined according to Hatchard and Parker [3]. The solution was 0.006 M in ferrioxalate. In the calculations the quantum yields 1.24 , 1.23 and 1.21 were used at the wavelengths 302 , 334 , and $365 \text{ m}\mu$ respectively. The optical density of the solution was measured in a Beckman DU spectrophotometer immediately after the exposure. The solutions were kept for six days to allow for a post-irradiation effect, and the viscosity was measured at different concentrations in a capillary viscosimeter of the Ubbelohde type.

At the wavelengths used, the solvent, methanol, has no absorption. A solution of nitrocellulose showed depolymerization after exposure to light of the wavelength $302 \text{ m}\mu$, whereas no depolymerization was detectable after exposure to the wavelengths 334 and $365 \text{ m}\mu$. Solutions containing β -naphthylamine showed depolymerization at all the wavelengths. Fig. 1 gives the extinction coefficient versus wavelength for cellulose nitrate and β -naphthylamine, in methanol. A continued rapid decrease in the viscosity is observed after the irradiation. The magnitude of this effect is seen in Fig. 2.

The graph shows the viscosity changes during the first 50 hours after the exposure. When the viscosity measurements were extended over a longer period (a couple of

Table 1.

Wavelength, 302 $m\mu$.Average light intensity, 2.8×10^{15} $h\nu$ /sec.

Concentration of cellulose nitrate, 0.4492 g/100 ml.

Exposure time, min	Incident dose, $h\nu$	Absorbed dose, $h\nu$	$[\eta]$, dl/g
0	—	—	4.14
8	1.5×10^{18}	0.45×10^{18}	4.03
16	3.1	0.93	3.93
71	11.8	3.54	3.16
210	27.8	8.34	2.41

Wavelength, 334 $m\mu$.Average light intensity, 3.6×10^{15} $h\nu$ /sec.

Concentration of cellulose nitrate, 0.4525 g/100 ml.

Concentration of β -naphthylamine, 4.92 mg/100 ml.

Exposure time, min	Incident dose, $h\nu$	Absorbed dose, $h\nu$	$[\eta]$, dl/g
0	—	—	4.14
10	2.04×10^{18}	1.8×10^{18}	4.10
20	2.87	2.5	4.09
25	8.55	7.6	3.96
210	33.9	31	3.50

Wavelength, 365 $m\mu$.Average light intensity, 2.7×10^{16} $h\nu$ /sec.

Concentration of cellulose nitrate, 0.4084 g/100 ml.

Concentration of β -naphthylamine, 5.44 mg/100 ml.

Exposure time, min	Incident dose, $h\nu$	Absorbed dose, $h\nu$	$[\eta]$, dl/g
0	—	—	4.14
5	7.5×10^{18}	4.4×10^{18}	4.04
10	17.9	10.5	3.90
20	30.0	18.4	3.78

weeks), it was shown that the effect soon became negligible, and the viscosity soon takes on a constant value. In order to achieve a measure of the total depolymerization, the irradiated solutions were kept for six days before the viscosity readings. No further effect on the viscosity was notable after this period. This post-effect was observed with all solutions.

The absorbed dose was set equal to the light absorbed by a 1.2 cm solution layer (the diameter of the cuvette) which involves only a slight approximation. The changes in absorption at the wavelength 302 $m\mu$ were found to be negligible for solutions only containing cellulose nitrate, and the light absorbed is directly obtained. For solutions containing β -naphthylamine a correction must be made for the changes in absorption at the wavelength of the actinic light. The absorbed dose is thus obtained through

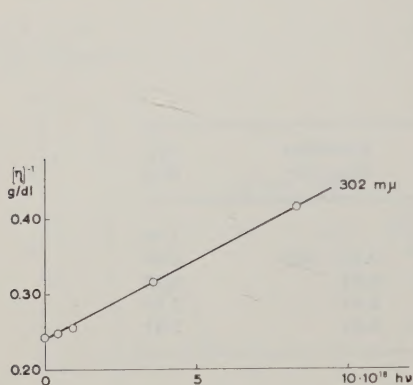


Fig. 3.

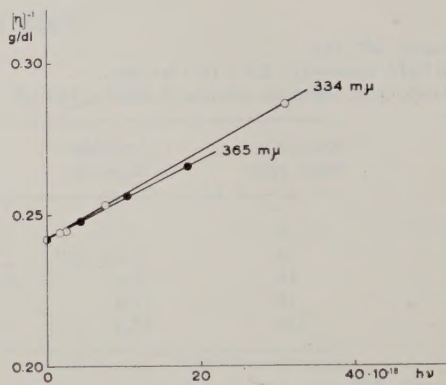


Fig. 4.

Fig. 3. $[\eta]^{-1}$ as a function of the absorbed dose for the degradation of cellulose nitrate with light of wavelength 302 $m\mu$.

Fig. 4. $[\eta]^{-1}$ as a function of the absorbed dose for the degradation of cellulose nitrate with light of wavelengths 334 and 365 $m\mu$. The light was absorbed by the sensitizer, β -naphthylamine.

graphical integration of curves with absorption as a function of the incident light dose. No corrections for scattered light have been made.

Table 1 shows the data from the irradiations. The intrinsic viscosity values have been obtained through straight line extrapolations to zero concentration.

The experimental data have been treated in the way described in an earlier paper [1]. The reciprocal of intrinsic viscosity was plotted as a function of the absorbed dose and is shown in Figs. 3 and 4. The quantum yield for the direct chemical action at the wavelength 302 $m\mu$, obtained from the slope of the straight line in Fig. 3 is 0.009. The quantum yield for the direct photochemical action is thus essentially the same for the wavelengths 253.7 and 302 $m\mu$.

For the sensitized degradation at the wavelengths 334 and 365 $m\mu$ the quantum yield was calculated from the lines in Fig. 4 to be 0.0006 in both cases. The quantum yield for the sensitized degradation is thus about one power of ten lower than for the direct photochemical action.

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